

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Hiroaki ITO *et al.*

Title: A PREVENTIVE OR THERAPEUTIC AGENT FOR INFLAMMATORY BOWEL DISEASE COMPRISING IL-6 ANTAGONIST AS AN ACTIVE INGREDIENT

Appl. No.: 10/677,227

Filing Date: 10/3/2003

Examiner: Prema Maria MERTZ

Art Unit: 1646

Confirmation Number: 8597

**DECLARATION OF BIOLOGICAL DEPOSIT**

Mail Stop Petition  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Simon J. Elliott, declare:

1. *Escherichia coli* containing the plasmid pIBIBSF2R was deposited on Jan. 9, 1989, with the Fermentation Research Institute Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. The deposit was assigned number FERM BP-2232.
  
2. The name and address of the depository institute has been changed to "the National Institute of Advanced Industrial Science and Technology, Patent and Bio-Resource Center, Chuo-6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-5466, Japan."

3. The deposit shall be made for a term of at least thirty (30) years, and at least five (5) years after the most recent request for the furnishing of a sample of the deposit was received by the depository. Samples will be stored under agreements that would make them available beyond the enforceable life of the patent for which the deposit was made. 37 C.F.R. § 1.806.

4. All restrictions on the availability to the public of the cultures deposited will be irrevocably removed upon the granting of a patent from the above-identified application. 37 C.F.R. § 1.808.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

Respectfully submitted,

Date February 22, 2010

By Simon J. Elliott

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Simon J. Elliott  
Attorney for Applicant  
Registration No. 54,083

# **EXHIBIT A**

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Format: GenBank FASTA Graphics More Formats▼

GenBank: X12830.1

## Human mRNA for interleukin-6 (IL-6) receptor

Comment	Features	Sequence
LOCUS X12830	3319 bp	mRNA linear PRI 07-OCT-2008
DEFINITION Human mRNA for interleukin-6 (IL-6) receptor.		
ACCESSION X12830 M20566		
VERSION X12830.1 GI:33845		
KEYWORDS BSF-2 receptor; glycoprotein; interferon beta II receptor; interleukin 6 receptor; receptor; transmembrane protein.		
SOURCE Homo sapiens (human)		
ORGANISM Homo sapiens		
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.		
REFERENCE 1 (bases 1 to 3319)		
AUTHORS Yamasaki,K., Taga,T., Hirata,Y., Yawata,H., Kawanishi,Y.,		
Seed,B.,		
TITLE Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor		
JOURNAL Science 241 (4867), 825-828 (1988)		
PUBMED 3136546		
REFERENCE 2 (bases 1 to 3319)		
AUTHORS Hirano,T.		
TITLE Direct Submission		
JOURNAL Submitted (05-SEP-1988) Hirano T., Division of Immunology, Institute for Molecular and Cellular Biology, Osaka University, -3		
COMMENT Yamada oka, Suita, Osaka 565, Japan		
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Change Region Shown  
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Analyze This Sequence  
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Pick Primers  
Articles about the IL6R gene  
Novel loci, including those related to Crohn Disease [see [Abstracts](#) ([2009](#))]  
[Association of the D358A polymorphism with [IL6R gene](#) ([2009](#))  
Genotype at the sIL-6R A358C polymorphism ([2010](#))  
» See all...

RefSeq Alternative Splicing  
See 2 reference mRNA sequence splice variants for the IL6R gene.

More about the IL6R gene  
Interleukin 6 (IL-6) is a potent pleiotropic cytokine that regulates cell growth and differentiation and plays an important role in immune re...  
Also Known As: CD126, IL-6R-1, IL-6R-a...

Homologs of the IL6R gene  
The IL6R gene is conserved in chimpanzee, dog, cow, mouse, and rat.

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M20566 (1)  
Human mRNA for interleukin-6 (IL-6) receptor  
X12830 (1) Nucleotide  
Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor precursor (AA -19 to 449)  
RecName: Full=Interleukin-6 receptor subunit alpha;  
» See more...

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GEO profiles

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# **EXHIBIT B**

Published October 1, 1987

**RECEPTORS FOR B CELL STIMULATORY FACTOR 2**  
**Quantitation, Specificity, Distribution, and Regulation of Their**  
**Expression**

BY TETSUYA TAGA, YOSHIKAZU KAWANISHI, RICHARD R. HARDY,  
 TOSHIO HIRANO, AND TADAMITSU KISHIMOTO

*From the Division of Immunology, Institute for Molecular and Cellular Biology,  
 Osaka University, Osaka 565, Japan*

Several factors are involved in the regulation of growth and differentiation of B cells (reviewed in 1). In fact, B cell stimulatory factor 2 (BSF-2),<sup>1</sup> which has recently been cloned (2), is a distinct cytokine of 21 kD that acts on activated normal human B cells as well as EBV-transformed B-lymphoblastoid cell lines to induce immunoglobulin secretion (3, 4). The study with the antipeptide antibody specific to BSF-2 demonstrated that several tumor cells, including cardiac myxomas, produce BSF-2, and patients with such tumors show hypergammaglobulinemia and autoantibody production (5). From these earlier investigations it was suggested that BSF-2 has an important role in the regulation of antibody production.

Recent reports have indicated identity between BSF-2 and other cytokines known as interferon  $\beta_2$  (IFN- $\beta_2$ ) and hybridoma plasmacytoma growth factor (HPGF) (6, 7). Another identical molecule is also reported as a 26 kD protein (8) that is expressed in human fibroblast by poly (IC) in the presence of cycloheximide (9) or (IL-1) (10). These facts suggest that BSF-2 has an important role not only in the immunoglobulin production of B cells but also in the regulation of growth and differentiation of several other types of cells. The recombinant BSF-2 had no detectable antiviral activity<sup>2</sup> (11), and it has been proposed that it be called IL-6 (11).

However, no information is available as to the presence of specific BSF-2 receptors (BSF-2-R) mediating the physiological effects. The availability of highly purified BSF-2 produced in *Escherichia coli* by recombinant DNA techniques enabled us to study the presence and the properties of BSF-2-R. We report here in this study the number and distribution of BSF-2-R. Our findings indicate that BSF-2-R are widely distributed in several tissues and cell lines. In contrast to BSF-1-R, which are found on resting B cells (12), our observations indicate that normal B cells express BSF-2-R only after activation.

<sup>1</sup> Abbreviations used in this paper: AET, S-(2-aminoethyl)isothiouronium bromide; BSF-2, B cell stimulatory factor 2; G-CSF, granulocyte colony-stimulating factor; MNC, mononuclear cell; SAC, *Staphylococcus aureus* Cowan I.

<sup>2</sup> Hirano, T., T. Matsuda, K. Hosoi, A. Okano, H. Matsui, and T. Kishimoto. B cell stimulatory factor 2 (BSF-2/IFN- $\beta_2$ ) does not belong to the family of interferons. Manuscript submitted for publication.

Published October 1, 1987

### Materials and Methods

**Lymphokines.** Recombinant BSF-2 was prepared by expressing a cDNA for BSF-2 (2) in *E. coli*, followed by further purification. Specific activity was determined as  $3.6 \times 10^9$  U/g by using the BSF-2-responsive human B lymphoblastoid cell line, SKW6-CL4 (4). Human IFN- $\beta$  and IFN- $\gamma$  were kind gifts from Toray, Co., Ltd. (Tokyo, Japan) and possessed specific activities of  $1.3 \times 10^{11}$  and  $5.9 \times 10^9$  U/g, respectively. Recombinant human IL-1 $\beta$  and IL-2 were supplied by Otsuka pharmaceutical Co., Ltd. (Tokushima, Japan), at specific activities of  $2.0 \times 10^{10}$  and  $5.0 \times 10^{10}$  U/g, respectively. Recombinant human granulocyte colony-stimulating factor (G-CSF) with a specific activity of  $2.5-10 \times 10^{10}$  U/g was generously provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

**Iodination of BSF-2.**  $^{125}\text{I}$ -BSF-2 was prepared as described by Bolton and Hunter (13) with some modifications. 5  $\mu\text{g}$  of recombinant BSF-2 dissolved in 10  $\mu\text{l}$  of 0.1 M borate buffer, pH 8.5, was added to 500  $\mu\text{Ci}$  of dry Bolton and Hunter reagent (2,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and the reaction mixture was agitated for 15 min on ice. The reaction was stopped by adding 0.5 mol of 0.2 M glycine in 0.1 M borate buffer, pH 8.5, with further mixing for 5 min on ice.  $^{125}\text{I}$ -labeled BSF-2 was separated from  $^{125}\text{I}$ -labeled products of low molecular mass using a gel filtration column (PD-10; Pharmacia Fine Chemicals, Piscataway, NJ).  $^{125}\text{I}$ -BSF-2 was diluted with RPMI 1640, 25 mM Hepes, pH 7.2, 3 mg/ml BSA, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 100 U/ml penicillin (binding medium), filter sterilized, and stored at 4°C. Purity and molecular mass of  $^{125}\text{I}$ -BSF-2 was examined by SDS-PAGE on a slab gel (10-20% continuous gradient of acrylamide) followed by autoradiography.

**Binding Assay.** Usual binding assay was performed as follows: cells for assay were washed twice and incubated in binding medium for at least 10 min at 37°C.  $10^6$  cells were mixed with  $^{125}\text{I}$ -BSF-2 with or without a 200-fold excess of unlabeled BSF-2 in a final volume of 70  $\mu\text{l}$  of binding medium, and incubated on ice for 150 min with occasional agitation every 10-15 min. At the end of incubation, the reaction mixture was layered on a 300- $\mu\text{l}$  cushion of FCS in a 400- $\mu\text{l}$  polypropylene tube and centrifuged at 9,000 rpm for 90 s. The tube was cut just above the cell pellet, and the cell-associated radioactivity was measured in a Beckman Gamma 9000 (Beckman Instruments, Fullerton, CA). The specific binding was calculated after subtracting the count of the samples with 200-fold excess unlabeled BSF-2 (nonspecific bound count).

**Determination of Specific Radioactivity of  $^{125}\text{I}$ -BSF-2.** Maximal binding capacity was measured according to a described method (14). Briefly, varying numbers of EBV-transformed B-LCL, CESS cells were mixed with 9,300 cpm of  $^{125}\text{I}$ -BSF-2 with or without a 200-fold excess of unlabeled BSF-2 in a final volume of 70  $\mu\text{l}$  of binding medium. Reaction mixtures were incubated on ice for 150 min with frequent agitation every 10-15 min. Specific binding was calculated as indicated above and data were plotted as reciprocal radioactivity specifically bound versus reciprocal cell number. From the ordinate intercept of a regression line of the plots, maximal binding capacity at infinite cell number was determined.

**Self-displacement analysis** was performed according to Calvo et al. (14).  $10^6$  CESS cells were mixed with 9,300 cpm of  $^{125}\text{I}$ -BSF-2 and increasing amounts of unlabeled BSF-2 or  $^{125}\text{I}$ -BSF-2 were added in a total volume of 70  $\mu\text{l}$  of binding medium. At the end of 150 min of incubation on ice, the cells were separated by centrifugation on FCS, and cell-associated radioactivity was measured. Data were transformed into a bound/free ratio and plotted against  $\log_{10}$ (amount of unlabeled BSF-2 [in nanograms]) or  $\log_{10}$ (amount of  $^{125}\text{I}$ -BSF-2 [counts per minute]). Two regression lines were drawn and the specific radioactivity was determined as the amount of radioactivity divided by the amount of unlabeled BSF-2 added to obtain the same bound/free ratio.

**Human Cell Lines.** SK-MG-4 and SK-N-MC were kindly provided by Dr. R. Ueda, Aichi Cancer Institute, Nagoya, Japan. Daudi, HSB, CEM, Jurkat, and U937 were provided by Dr. P. Ralph, Memorial Sloan-Kettering Cancer Center, New York. Reh and KM-3 were provided by K. Kikuchi, Sapporo Medical College, Sapporo, Japan. HL60 and U937 were obtained from the American Type Culture Collection, Rockville, MD. BL41 and BL41/45 were from Dr. G. Klein (Karolinska Institute). Louckes and EBNA2-

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969

200	—
92.5	—
69	—
46	—
30	—
14.3	—

FIGURE 1. Autoradiograph of  $^{125}\text{I}$ -BSF-2 analyzed by SDS-PAGE. Recombinant BSF-2 was iodinated and an aliquot was applied on polyacrylamide gel with a continuous gradient of 10–20% acrylamide. The molecular mass (kD) of protein standards are also indicated.

transfected Louckes cells were from Dr. E. Kieff (University of Chicago, Chicago, IL). EBV-transformed B cell lines SKW6-CL4, LCL18, and LCL14 were described elsewhere (2, 15). All human cell lines except U373 were maintained in RPMI 1640, 10% FCS,  $5 \times 10^{-5}$  M 2-ME, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin. U373 was cultured in MEM, 10% FCS, 100  $\mu\text{g}/\text{ml}$  sodium pyruvate, and nonessential amino acid mixture (Gibco, Grand Island, NY). All were cultured at 37°C, in humidified 5% CO<sub>2</sub> in air.

**Normal Human T and B Cells.** Human T and B cells were prepared as follows. Tonsillar cells were dispersed on a plastic dish and mononuclear cells (MNC) were isolated by centrifugation on Ficoll-paque (Pharmacia Fine Chemicals). T cells were prepared from MNC by two sequential rosettes with S-(2-aminoethyl)isothiouronium bromide (AET)-treated SRBC. B cells were isolated from MNC by AET-treated SRBC-rosetting twice, and rosette-negative cells were further treated with anti-CD2 (kindly provided by Dr. E. L. Clark) and rabbit complement to eliminate possible contaminating T cells. The purity of T and B cells were examined by FACS analysis. T and B cells were cultured in the same medium described above for most of the cell lines at a density of  $2 \times 10^6/\text{ml}$ . PWM (Sigma Chemical Co., St. Louis, MO), 2.5  $\mu\text{g}/\text{ml}$  final concentration; PHA (Sigma Chemical Co.), 0.1% wt/vol, final concentration; and *Staphylococcus aureus* Cowan I (SAC) from Bethesda Research Laboratories, Gaithersburg, MD, 0.005% (vol/vol), final concentration, were used as stimulants. Size fractionation of B and T cells was performed by Percoll (Pharmacia Fine Chemicals) discontinuous gradient centrifugation. Large cells were separated from the interface between 40 and 50% Percoll, and small cells were from the interface between 55 and 70% after centrifugation at 3,500 rpm for 25 min at 4°C.

### Results

**Kinetics of Binding of  $^{125}\text{I}$ -BSF-2.** Recombinant BSF-2 was radiolabeled with  $^{125}\text{I}$  as described in Materials and Methods, and radioiodinated BSF-2 was analyzed by SDS-PAGE. Autoradiography showed only one major band, with an  $M_r$  of 21,000 (Fig. 1). This preparation was used for the analysis of BSF-2-R.

The EBV-transformed B-LCL, CESS, which is known to be responsive to BSF-2 (16), was used for the initial binding studies. As shown in Fig. 2, the binding of BSF-2 at 0°C achieved a steady-state level within 150 min. Maximum binding at 37°C was about one-half that observed at 0°C. The addition of 0.02% sodium azide could keep the binding stable at 37°C, but could not increase the maximum

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## 970 RECEPTORS FOR B CELL STIMULATORY FACTOR 2

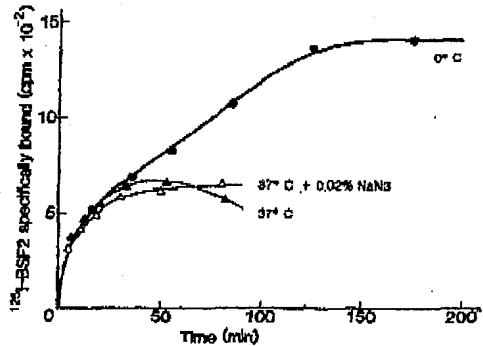


FIGURE 2. Kinetics of  $^{125}\text{I}$ -BSF-2 binding to CESS cells,  $10^6$  CESS cells in 70  $\mu\text{l}$  binding medium with 11,000 cpm  $^{125}\text{I}$ -BSF-2 in the presence or absence of 200-fold excess unlabeled BSF-2 were incubated for the indicated times at 0°C or 37°C with or without 0.02% sodium azide. Data represent the mean specific binding of duplicate samples with the deviation usually <5%.

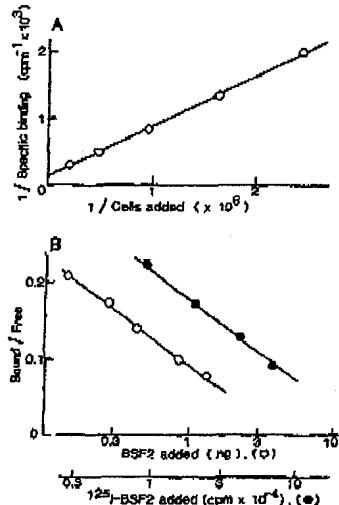


FIGURE 3. Determination of the maximal binding capacity and specific radioactivity of  $^{125}\text{I}$ -BSF-2. (A) 9,800 cpm of  $^{125}\text{I}$ -BSF-2 was incubated for 150 min with five different concentrations of CESS cells (up to  $5 \times 10^6$ ) in the presence or absence of 200-fold excess amount of unlabeled BSF-2. Data (averages of duplicates) are plotted as reciprocal specific binding versus reciprocal cells added. The ordinate intercept of the regression line means the reciprocal counts that would bind at infinite number of cells (maximal binding capacity). (B) Self-displacement analysis is performed as described in Materials and Methods. 9,800 cpm of  $^{125}\text{I}$ -BSF-2 and increasing amounts of unlabeled BSF-2 (up to 1.8 ng) or  $^{125}\text{I}$ -BSF-2 (up to 80,000 cpm) were incubated with  $10^6$  CESS cells. The mean specific binding of duplicates is plotted as a bound/free ratio (corrected for maximal binding capacity). The specific radioactivity is determined as mentioned in the text.

binding. In addition, the level of the nonspecific binding of  $^{125}\text{I}$ -BSF-2 at 37°C in the presence of 200-fold excess unlabeled BSF-2 was twofold as high as that at 0°C (data not shown). Therefore, the standard experimental condition of 150 min incubation at 0°C, was used through the study.

**Determination of Specific Radioactivity of  $^{125}\text{I}$ -BSF-2.** To determine how much of the total radiolabeled BSF-2 was bindable to CESS, we used a graphic method presented previously (14). As briefly described in Materials and Methods, increasing numbers of CESS cells were incubated with a constant amount of  $^{125}\text{I}$ -BSF-2 in a final volume of 70  $\mu\text{l}$ . Nonspecific binding of  $^{125}\text{I}$ -BSF-2 was measured by the addition of 200-fold excess of unlabeled BSF-2. Fig. 3A shows the regression line of the plot of reciprocal specific binding versus reciprocal cell number. From its ordinate intercept, the maximal binding capacity at the infinite cell number

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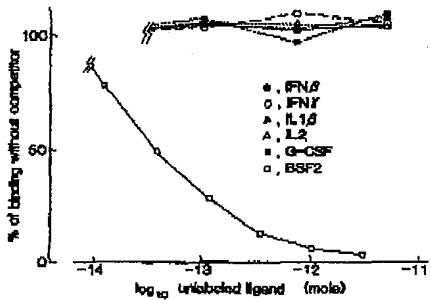


FIGURE 4. Competition for the binding of  $^{125}\text{I}$ -BSF-2 by unlabeled BSF-2 and other lymphokines to CESS cells.  $10^6$  CESS cells and  $5.7 \times 10^{-10}$  mol of  $^{125}\text{I}$ -BSF-2 are incubated in 70  $\mu\text{l}$  binding medium on ice for 150 min with indicated amount of lymphokines. Data are mean of duplicate samples.

was calculated to be 82.6% of the total radioactivity. This means only 17.4% of the initial BSF-2 lost its binding ability during radioiodination.

The specific radioactivity of  $^{125}\text{I}$ -BSF-2 was determined by self-displacement analysis (14). Details are described in Materials and Methods. Fig. 3B shows two regression lines of bound/free ratio versus additional amount of unlabeled BSF-2 or additional  $^{125}\text{I}$ -BSF-2 to a constant set of initial  $^{125}\text{I}$ -BSF-2 and CESS cells. The identical slope of the two lines indicates that there was no significant difference in binding affinity between unlabeled BSF-2 and  $^{125}\text{I}$ -BSF-2 to its receptors. The results indicate that  $2.89 \times 10^4$  cpm of  $^{125}\text{I}$ -BSF-2 was equivalent to 0.986 ng of BSF-2 in the meaning of the induction of the same bound/free ratio of 0.15, thus the calculated specific radioactivity (corrected for the maximal binding capacity) of  $^{125}\text{I}$ -BSF-2 was  $6.16 \times 10^{13}$  cpm/g.

**Specificity of  $^{125}\text{I}$ -BSF-2 Binding on CESS Cells.** The ability of unlabeled BSF-2 and other cytokines to compete with  $^{125}\text{I}$ -BSF-2 for binding sites on CESS cells is shown in Fig. 4. Unlabeled BSF-2 inhibited the binding of  $^{125}\text{I}$ -BSF-2 in a dose-dependent manner. As much as 96.5% of the total radioactivity of  $^{125}\text{I}$ -BSF-2 bound to CESS without competitor was inhibited by 560-fold excess unlabeled BSF-2. No significant competition was observed with any other cytokines, such as IFN-β, IFN-γ, IL-1β, IL-2, and G-CSF. Previous studies had shown sequence homology between BSF-2, G-CSF (2), and IFN-β (17). However, large excess of G-CSF or IFN-β could not inhibit the binding of  $^{125}\text{I}$ -BSF-2 to CESS, indicating that BSF-2, IFN-β, and G-CSF are using different receptors.

**Scatchard Plot Analysis of Binding of  $^{125}\text{I}$ -BSF-2.** The specific binding of  $^{125}\text{I}$ -BSF-2 to CESS cells as a function of the concentration of  $^{125}\text{I}$ -BSF-2 is shown in Fig. 5A.  $^{125}\text{I}$ -BSF-2 bound to CESS in a saturable manner. Analysis of the binding data by the method of Scatchard (18) revealed only one linear regression line, indicating that there was a single set of binding sites on CESS (Fig. 5B). The negative inverse of the regression coefficient gave a dissociation constant of  $3.4 \times 10^{-10}$  M and the abscissa intercept provided a number of 2,700 binding sites per cell.

**BSF-2-R on Human Cell Lines.** Scatchard analyses were carried out on various human cell lines and are summarized in Table I. All the analyzed EBV-transformed B cell lines had BSF-2-R with an affinity of  $2.0\text{--}4.0 \times 10^{-10}$  M. The number of receptors on B-LCL was in the range of 200–2,700 per cell. In contrast, none of the Burkitt's lymphoma cell lines were found to express any

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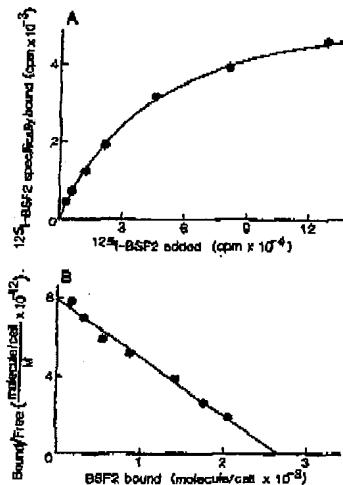


FIGURE 5. (A) Specific binding of  $^{125}\text{I}$ -BSF-2 to EBV-transformed B cell line CESS. (B) Scatchard plot of the same data. The average of the duplicate is shown.

detectable number of receptors. The infection of EBV into EBV<sup>+</sup> Burkitt's cells (BL41) or the transfection of EBNA2 into Burkitt's cells (Louckes) (19) did not induce any expression of the BSF-2-R. The plasma cell lines ARH-77 and U266 displayed 120 and 11,000 receptors per cell, respectively, with affinities of  $1.5 \times 10^{-10}$  and  $2.5 \times 10^{-10}$  M. Four T cell lines tested had no BSF-2-R.

BSF-2-R levels equivalent to CESS cells were detectable on histiocytic leukemia cell line U937 and promyelocytic leukemia HL60, whereas the receptor number on astrocytoma U378 and glioblastoma SK-MG-4 was in the low detectable range. Several other hematopoietic cell lines, such as a non-T, non-B line, KMS and Reh, did not express any receptors.

**BSF-2-R on Normal Lymphocytes.** Normal human T and B cells were purified from tonsillar mononuclear cells and examined for the expression of BSF-2-R. Fig. 6 shows specific binding of  $^{125}\text{I}$ -BSF-2 to human T cells and its Scatchard analysis. The results showed that resting T cells expressed BSF-2-R with a homogeneous affinity. The binding sites per cell were around 300 and the affinity was  $1.4 \times 10^{-10}$  M. Upon stimulation of T cells for 3 d with 0.1% PHA or 0.003% (vol/vol) SAC + 2.5  $\mu\text{g/ml}$  PWM, the level of binding sites remained constant or were slightly decreased. Activated large T cells displayed fewer receptors than the smaller ones. These results, summarized in Table II, are in contrast to the fact that four human T cell lines examined had no significant number of BSF-2-R (Table I).

In a marked contrast, freshly prepared resting human B cells displayed no detectable BSF-2-R in the absence of stimulation (tonsils 1, 2, and 3 in Table II, lower half). The level of receptor number increased upon activation of B cells with 0.003% SAC for 1, 2, or 3 d (tonsils 3, 4, and 6). The result obtained with B cells from the tonsil of individual 3 showed that B cells without any detectable BSF-2-R before stimulation were induced to express the receptors after 3 d of culture with SAC, but not with medium alone. Another result with tonsillar B

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TABLE I  
*Characteristics of BSF-2-R on Human Cell Lines*

Cell line	Cell type	Sites per cell	$K_d$ $\times 10^{-10} M$
CESS	EBV-B	2,700	3.4
SKW6-CL4	EBV-B	210	2.0
LCL13	EBV-B	310	4.0
LCL14	EBV-B	410	3.9
BL29	Burkitt's	NS*	—
BL36	Burkitt's	NS	—
BL41	Burkitt's	NS	—
BL41/95	Burkitt's (EBV-infected)	NS	—
Daudi	Burkitt's	NS	—
Raji	Burkitt's	NS	—
Louckes	Burkitt's	NS	—
Louckes/EBNA2	Burkitt's (EBNA2-transfected)	NS	—
ARH-77	Plasma cell	120	1.5
U266	Plasma cell	11,000	2.5
CEM	T	NS	—
HSB	T	NS	—
Jurkat	T	NS	—
OM1	T	NS	—
KM8	Non-T non-B	NS	—
Reh	Non-T non-B	NS	—
U937	Histiocytoma	2,800	3.3
HL60	Promyelocytic leukemia	3,600	6.4
U373	Astrocytoma	170	1.5
T24	Bladder carcinoma	NS	—
SK-MG-4	Glioblastoma	150	8.9
SK-N-MC	Neuroblastoma	NS	—

Cells were analyzed by usual binding assay (see Materials and Methods) and a Scatchard plot. At least four different doses of  $^{125}\text{I}$ -BSF-2 were used. The averages of duplicate determinations were taken for analysis. BL41/95 (27) is an EBV-infected cell line originated from a Burkitt's lymphoma cell line, BL41.

\* Not significant (<30).

cells from individual 4 demonstrated that activated larger B cells on day 1 of stimulation with SAC expressed ~600 binding sites per cell with a  $K_d$  of  $4.2 \times 10^{-10} M$ , whereas smaller B cells displayed barely detectable number of receptors (120 receptors), that was similar to the number before stimulation (see also Fig. 7). The result with the tonsil of individual 5 also demonstrated that freshly prepared large B cells which could be considered in vivo-activated cells, expressed a small but substantial number of BSF-2-R. Freshly prepared small resting B cells devoid of BSF-2-R (tonsil 6) were shown to express BSF-2-R after activation for 2 d with SAC, compared to unstimulated controls. The above results indicate that BSF-2-R are expressed constitutively on resting T cells, but are inducible on B cells.

**FACS Analysis of Normal T Cells and B Cells.** To know the purity of T cells and B cells prepared from tonsillar mononuclear cells, cells were stained with fluorescein-conjugated anti-CD3 or anti-CD20 and examined by FACS. The T

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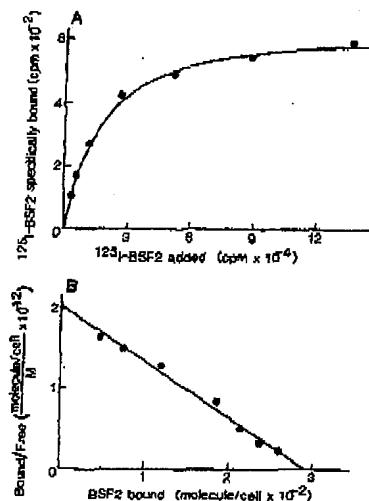


FIGURE 6. (A) Specific binding of  $^{125}\text{I}$ -BSF-2 to normal human T cells. (B) A Scatchard plot of the data in A. Data represent the mean of the duplicate.

cell fraction used in these studies contained >96% T cells and the B cell fraction consisted practically of 100% B cells (data not shown). To demonstrate that BSF-2-R were expressed mainly on the final maturation stage of B cells, large and small B cell fractions were stained with anti-Ba and anti-IgD antibodies (20). As shown in Fig. 8, a large B cell fraction, which expressed BSF-2-R, consisted mainly of  $\text{Ba}^{++}/\text{IgD}^-$  or  $\text{Ba}^-/\text{IgD}^-$  cells, while large portion of smaller B cell fraction was IgD<sup>+</sup>.

#### Discussion

Cloning of the cDNA for BSF-2 (2) enabled us to obtain highly purified BSF-2 protein generated in *E. coli* by recombinant DNA technology and has made it possible to study receptors for BSF-2. We have found that an efficient radioiodination of BSF-2 without hindering its binding ability can be achieved with the use of the Bolton and Hunter reagent. In fact, 88% of the iodinated BSF-2 retained its binding ability, with a specific radioactivity of  $6.16 \times 10^{13}$  cpm/g (Fig. 3). This material has allowed us to analyze the binding properties of BSF-2 and to detect the presence of very low number of receptors per cell. In fact, the identical slope of the two regression lines obtained by the addition of excess  $^{125}\text{I}$ -labeled or unlabeled BSF-2 (Fig. 3B) indicates that there was no significant difference in the binding affinity between labeled and unlabeled BSF-2 to its receptor. Native BSF-2 purified from a bladder carcinoma line, T24 (21), also gave a regression line parallel to those in Fig. 3B (data not shown). The molecular mass shown in Fig. 1 agrees well with the one of native BSF-2 shown by a previous study (5). Because the binding property of recombinant BSF-2 does not seem to be different from native BSF-2, it seems that glycosylation does not play a critical role in the binding of this cytokine to its receptor.

The kinetic study has demonstrated that binding of  $^{125}\text{I}$ -BSF-2 to CESS cells

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TABLE II  
*Characteristics of BSF-2-R on Normal Human T Cells and B Cells*

Cell type	Tissue (tonsil) from subject	Cell size*	Stimulant	Culture duration	Sites per cell†	$K_d$
T cells	1	—	—	0	290	1.4
	1	Large	0.1% PHA	3	220	—
	1	Small	0.1% PHA	3	310	1.5
	2	—	—	0	310	2.0
	2	Large	0.1% PHA	3	120	1.6
	2	Small	0.1% PHA	3	280	1.5
	3	—	—	0	350	1.4
	4	—	—	0	990	2.1
	4	—	PWM + SAC	3	270	2.8
B cells	1	—	—	0	NS	—
	2	—	—	0	NS	—
	3	—	—	0	NS	—
	3	—	Medium alone	3	NS	—2
	3	—	0.008% SAC	3	80	8.6
	4	—	—	0	70	4.5
	4	Large	0.008% SAC	1	570	4.9
	4	Small	0.008% SAC	1	120	5.0
	5	Large	—	0	87	4.2
	5	Small	—	0	NS	—
	6	Small	—	0	NS	—
	6	Unfractionated‡	—	2	NS	—
	6	Unfractionated‡	0.008% SAC	2	290	5.5

T cells and B cells were separated from tonsils of six individuals (numbered 1-6) and cultured with 0.1% PHA or 2.5 µg/ml PWM + 0.008% SAC (T cells), or 0.008% SAC or medium alone (B cells) at an initial density of  $2 \times 10^6$  cells/ml for the indicated duration. In some cases, cells were fractionated by size. Scatchard analyses were performed (see legend of Table I).

\* —, unfractionated.

† NS, not significant (<30).

‡ Freshly prepared small B cells with no detectable number of BSF-2-R were cultured in the absence of size-fractionation on the day of assay.

was saturated in 150 min at 0°C. In comparison, at 37°C the saturation was achieved in 60 min but the maximum level of binding was half that found at 0°C. This level was almost the same in the presence of 0.02% sodium azide at 37°C, although the maximum level was more stable than without sodium azide. Unlike other cytokines (22, 23), the binding property of BSF-2 to its receptor has a certain peculiarity, in that the level of binding at 0°C is higher than that at 37°C. In any case, as the nonspecific binding at 0°C was much lower than that at 37°C, the assay at 0°C provided a suitable condition for the measurement of BSF-2-R.

We have previously mentioned that the primary structure of BSF-2 has some homology at the NH<sub>2</sub>-terminal region with G-CSF (2), and that the organization of the genomic genes for BSF-2 and G-CSF, both of which had five exons and four introns, are almost identical (24). Although it has been suggested that some

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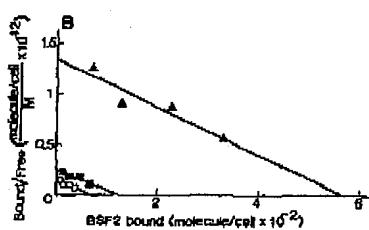
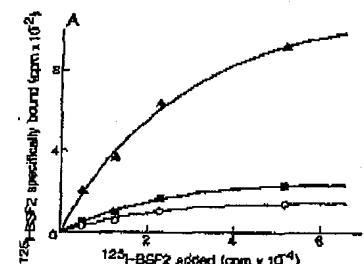


FIGURE 7. (A) Specific binding of  $^{125}\text{I}$ -BSF-2 to normal B cells. B cells were purified from tonsillar mononuclear cells and cultured with 0.009% SAC for 1 d and fractionized into large (closed triangles) and small (closed squares) cells by centrifugation on discontinuous gradient of Percoll. Unstimulated nonfractionated B cells (open circles) are also used for binding assay. (B) Scatchard plots of the same data.

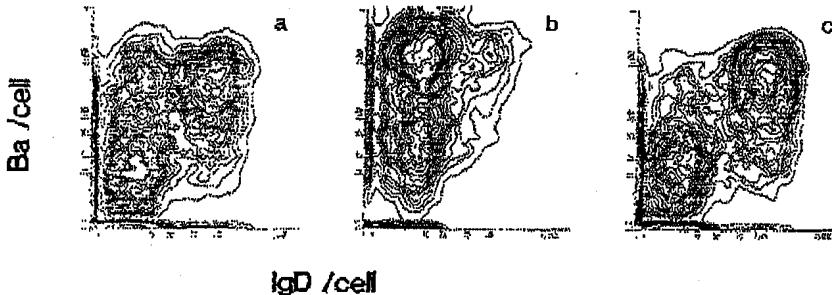


FIGURE 8. Two-color FACS analysis of tonsillar B cells stained with anti-Ba and anti-IgD. Freshly prepared tonsillar B cells (unfractionated, a; large, b; and small, c) were stained with FITC-anti-IgD and biotinylated anti-Ba, and developed with Texas Red-avidin. The expression of Ba and IgD on cells were analyzed by FACS 440.

similarity exists between BSF-2 and IFN- $\beta$  in the COOH-terminal portion (17), the present study clearly demonstrates that BSF-2 receptors can only bind BSF-2, not other cytokines such as IFN- $\alpha$  and G-CSF.

The presence of high- and low-affinity receptors for IL-2 and several other growth factors has been demonstrated (25, 26). From the binding profile and the Scatchard analysis (Fig. 5), only a single class of BSF-2 receptors was detected on CESS cells numbering 2,700 and having a dissociation constant of  $3.4 \times 10^{-10}$  M. Several cell lines possessed BSF-2-R with similar dissociation constants in the range of  $1.9-6.4 \times 10^{-10}$  M (Table I). Because  $1.2 \times 10^{-9}$  M BSF-2 was used at the maximum concentrations in these binding studies, the existence of another class of receptors with much lower affinity could not be completely excluded.

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The number of receptors was found to vary in the cell lines tested, ranging from 120 to 11,000. Considering the requirement of a very low amount ( $\sim 10^{-14}$  mol) of BSF-2 for the induction of 50% maximum response of SKW6-CL4 (10,000 cells in 200  $\mu$ l medium), which expressed only 210 receptors, partial occupancy of low numbers of BSF-2-R expressed on EBV-transformed B cells may be sufficient to be functional. Therefore, the presence of a large number of receptors might not be necessary for BSF-2 signal transduction, as observed in IL-1 stimulation (27).

It is noteworthy that BSF-2-R were expressed on all EBV-transformed B cell lines examined here but not on any Burkitt's lines (Table I). Two possible explanations could be given: (a) EBV induces B cells to express BSF-2-R; or (b) EBV can cause the arrest of B cells at a specific stage of growth or differentiation in which BSF-2-R could be expressed. The results shown in Table I using two sets of two different variants of the Burkitt's lines, BL41 or Louckes, showed that these lines did not express detectable BSF-2-R even after EBV infection or EBNA2 transfection. EBV-infected BL41/95 has been reported to express lymphoblastoid cell associated antigens, whereas BL41 did not (28), and we also observed BL41/95 making clumps in culture, a typical characteristic of EBV-infected B cells. This also shows a marked contrast to the expression of a B cell-specific differentiation antigen, CD28 (Fc $\epsilon$  receptor), the expression of which could be inducible by the transfection of EBNA2 (19). This result implies that the former of the two possible explanations described above is probably not correct, but further study is required to draw any firm conclusions on the mechanism of the regulation of BSF-2-R expression.

Not only EBV-transformed B cell lines but also various other human cell lines expressed BSF-2-R. Among them, it should be noted that human plasma cell lines, ARH-77 and U266, expressed BSF-2-R, since several investigators reported that BSF-2 could function as a plasmacytoma growth factor in a murine system (7, 29). In a preliminary study, recombinant BSF-2 could induce the proliferation of myeloma cells freshly prepared from a patient with myeloma (our unpublished data). Besides B cell lines and plasma cell lines, the histiocytic line U937, the promyelocytic line HL60, the astrocytoma line U373, and the glioblastoma line SK-MG-4 displayed BSF-2-R. The finding that BSF-2-R are expressed on cell lines of different types is in agreement with the wide range of target cells for BSF-2 (4, 6, 30, 31). Further study should clarify the function of BSF-2 on those receptor-positive cells. It is interesting to note that in the four cell lines described above, including an astrocytoma and a glioblastoma line, BSF-2-R were expressed and BSF-2 itself was inducible after stimulation with IL-1 or TPA (24 and our unpublished data), whereas a neuroblastoma line neither expressed BSF-2-R nor produced BSF-2. These results may suggest the presence of an autocrine mechanism in BSF-2-induced differentiation or growth of various cells.

One of the interesting findings was the expression of BSF-2-R on resting T cells. The situation is similar with BSF-1 (IL-4), which can act on T cells to induce their proliferation (32). Fewer BSF-2-R were detected on large T cells compared to small-size T cell fractions (Table II). This might explain the lack in BSF-2-R on the T cell lines examined, as seen in Table I. The role of BSF-2 on

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normal T cells is, as yet, unknown. But preliminary studies suggest that BSF-2 could induce IL-2-R expression on certain T cell lines (38).

Recent studies on receptors for cytokines such as IL-1, IL-2, BSF-1, and TNF (12, 34, 35, 36) demonstrated that cytokines do not have strict target cell specificity. The present studies show that BSF-2 is no exception. In such a situation, studies on the regulation of the expression of both cytokines and receptors are essential for the elucidation of the biological role of these molecules. Normal human B cells usually do not express a significant number of receptors for BSF-2 before activation (Table II). Upon activation with SAC they were observed to express 80–570 BSF-2 receptors per cell, with a  $K_d$  of  $4\text{--}5 \times 10^{-10}$  M. This smaller number of the receptors may be due to a smaller proportion of receptor-positive cells among total B cells. Large B cells after 1 d stimulation with SAC displayed more receptors than smaller ones. As shown in Table II, even before stimulation *in vitro*, large B cells freshly prepared from a tonsil expressed 87 receptors per cell with a similar  $K_d$  to other cases, whereas smaller B cells did not. This means normal B cells can express BSF-2-R when they are activated *in vivo*. As reported previously (20), by using anti-Ba (a monoclonal antibody that recognizes activated B cells) and anti-IgD, tonsillar B cells were separated into four subpopulations with regard to their activation stage; Ba<sup>+</sup>/IgD<sup>+</sup>, Ba<sup>+</sup>/IgD<sup>+</sup>, Ba<sup>++</sup>/IgD<sup>-</sup>, and Ba<sup>++</sup>/IgD<sup>-</sup>. As shown in Fig. 8, more than half of the freshly prepared large B cells were Ba<sup>++</sup>/IgD<sup>-</sup> and most cells were IgD<sup>-</sup>. This phenotype of B cell is known to be at the final maturation stage and ready to produce immunoglobulin (37). These results suggest that normal B cells become ready to respond to BSF-2 by expressing receptors only when they are activated. This is in contrast to the BSF-1-R, which are reported to exist on resting B cells (12, 38, 39). The difference between the mode of the expression of BSF-1- and BSF-2-R fits the functional difference between BSF-1 and BSF-2 on B cells; the former acts mainly on resting B cells to lead to proliferation in the presence of anti-IgM, and the latter acts on activated B cells to secrete immunoglobulin (4, 40). It is not necessary for normal B cells to have constitutive expression of the BSF-2-R, but only their transient expression might be sufficient to drive the cells to a final stage of differentiation.

## Summary

B cell stimulatory factor 2 receptors (BSF-2-R) were studied using radioiodinated recombinant BSF-2 with a specific activity of  $6.16 \times 10^{13}$  cpm/g. Kinetic studies showed that binding of  $^{125}\text{I}$ -BSF-2 to CESS cells reached maximum level within 150 min at 0°C. There was a single class of receptors with high affinity ( $K_d 3.4 \times 10^{-10}$  M) on CESS, and the number of receptors was 2,700 per cell. Binding of  $^{125}\text{I}$ -BSF-2 to CESS was competitively inhibited by unlabeled BSF-2 but not by IL-1, IL-2, IFN- $\beta$ , IFN- $\gamma$ , and G-CSF, indicating the presence of the receptors specific for BSF-2. EBV-transformed B lymphoblastoid cell lines (CESS, SKW6-CL4, LCL13, and LCL14) expressed BSF-2-R, whereas Burkitt's lines did not. EBV or EBNA2 did not induce the expression of the receptors on Burkitt's cells. The plasma cell lines (ARH-77 and U266) expressed BSF-2-R, fitting the function of BSF-2 as plasma cell growth factor. Several other cell lines, the histiocytic line U937, the promyelocytic line HL60, the astrocytoma line

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U973 and the glioblastoma line SK-MG-4, in which BSF-2 was inducible with IL-1 or TPA, displayed BSF-2-R with  $K_d$  in the range of  $1.3\text{--}6.4 \times 10^{-10}$  M, suggesting the autocrine mechanism in BSF-2 function. The four T cell lines (CEM, HSB, Jurkat, and OM1) did not express a detectable number of receptors, but normal resting T cells expressed 100–1,000 receptors per cell. BSF-2-R were not present on normal resting B cells but expressed on activated B cells with a  $K_d$  of  $3.6\text{--}5.0 \times 10^{-10}$  M, fitting the function of BSF-2, which acts on B cells at the final maturation stage to induce immunoglobulin production.

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# **EXHIBIT C**

chum measurements support this view (10). In normal cells, both the SR and  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange will contribute to relaxation by removing  $\text{Ca}^{2+}$  from the cytosol. However, the  $\text{Na}^+$ - $\text{Ca}^{2+}$  will be effective only when membrane potential ( $E_m$ ) is negative to the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange reversal potential ( $E_{rev}$ ). Owing to the inferred voltage dependence of  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange,  $\text{Ca}^{2+}$  extrusion increases as  $E_m$  becomes increasingly negative to  $E_{rev}$ . Thus, during a single twitch the trajectory of the quantity  $E_m - E_{rev}$  will determine both the onset and variation of  $\text{Ca}^{2+}$  efflux with time.

The foregoing facts will have important consequences for the regulation of contraction in heart muscle. If  $\text{Ca}^{2+}$  extrusion is abruptly delayed or reduced by prolonged membrane depolarization (for example, a prolonged action potential in which  $E_m$  spends less time negative to  $E_{rev}$ ), the SR could sequester  $\text{Ca}^{2+}$  normally removed by the exchanger. Alternatively, accumulation of internal  $\text{Na}^+$  as a result of glycoside applications would collapse  $E_m - E_{rev}$ , thereby reducing  $\text{Ca}^{2+}$  extrusion via the exchanger, with resulting increases in the SR  $\text{Ca}^{2+}$  pool. This enlarged SR  $\text{Ca}^{2+}$  store would presumably strengthen the subsequent contraction. In contrast, a brief depolarization (for example, shortened action potential) would have the opposite effect. Thus, the trajectory of  $E_m - E_{rev}$  can regulate competition between the SR and  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange for cytosolic  $\text{Ca}^{2+}$ . This suggests voltage-dependent control of sarcolemmal  $\text{Ca}^{2+}$  extrusion via  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange can provide an effective and delicate mechanism for regulating the SR  $\text{Ca}^{2+}$  available for contraction.

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Cloning and Expression of the Human Interleukin-6 (BSF-2/IFN $\beta$  2) Receptor

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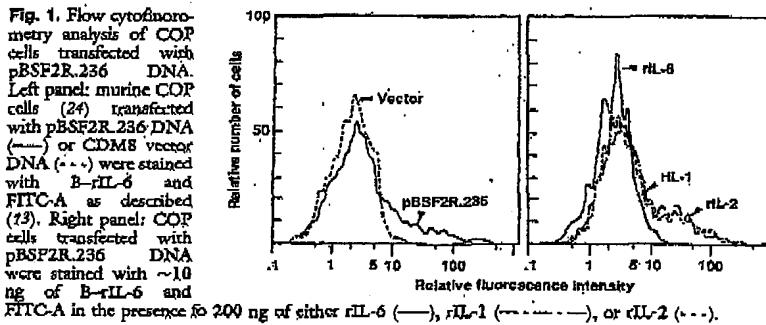
Interleukin-6 (IL-6/BSF-2/IFN $\beta$  2) is a multifunctional cytokine that regulates the growth and differentiation of various tissues, and is known particularly for its role in the immune response and acute phase reactions. A complementary DNA encoding the human IL-6 receptor (IL-6-R) has now been isolated. The IL-6-R consists of 468 amino acids, including a signal peptide of ~19 amino acids and a domain of ~90 amino acids that is similar to a domain in the immunoglobulin (Ig) superfamily. The cytoplasmic domain of ~82 amino acids lacks a tyrosine/kinase domain, unlike other growth factor receptors.

**B** CELL STIMULATORY FACTOR-2 (BSF-2) was originally identified as a T cell-derived factor that causes the terminal maturation of activated B cells to Ig-producing cells (1). After the cDNAs were cloned, BSF-2 was found to be identical to the 26-kD protein, IFN- $\beta$  2, myeloma-plasmacytoma growth factor and hepatocyte stimulating factor (2-6). It is established that BSF-2, now called IL-6, has

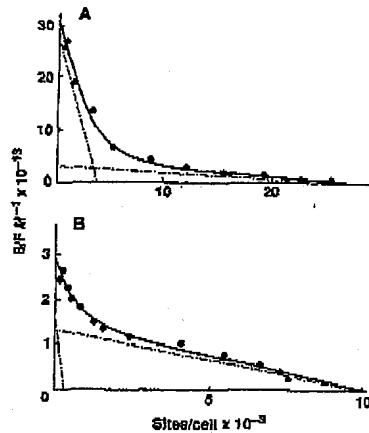
many biological functions, which include growth and differentiation activities on B cells (1, 2, 7), T cells (8), myeloma-plasma-

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**Fig. 1.** Flow cytometry analysis of COP cells transfected with pBSF2R.236 DNA. Left panel: murine COP cells (24) transfected with pBSF2R.236 DNA (—) or CDM8 vector DNA (---) were stained with B-IL-6 and FITC-A as described (13). Right panel: COP cells transfected with pBSF2R.236 DNA were stained with ~10 ng of B-IL-6 and FITC-A in the presence of 200 ng of either rIL-6 (—), rIL-1 (---), or rIL-2 (---).



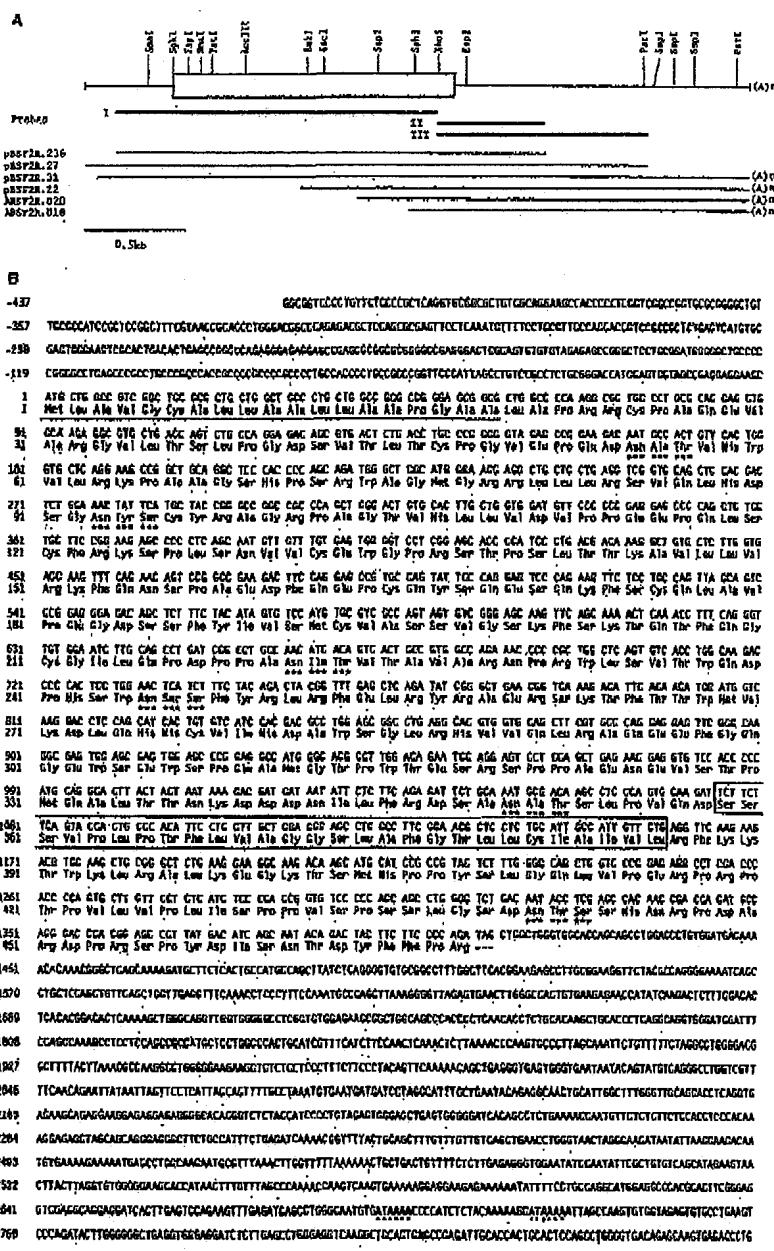
cytomas (4, 5, 9), hepatocytes (6), hematopoietic stem cells (10), and nerve cells (11).

To elucidate how one cytokine can mediate multiple functions, the structure of their receptor molecules must be determined. However, the low number of cytokine receptors on target cells ( $10^2$  to  $10^3$  per cell) makes their isolation and characterization difficult. We now report the cloning of the cDNA for IL-6 receptor (IL-6-R) utilizing a high-efficiency COS7 cell expression system with the CDM8 vector (12). The expressed receptors were detected with biotinylated-recombinant IL-6 (B-rIL-6) and fluorescein-conjugated avidin (FITC-A).

We constructed a cDNA library from polyadenylated [poly(A)<sup>+</sup>] RNA of a human NK-like cell line, YT (12). Plasmid DNA was transfected into monkey COS7 cells, and the cells were stained with B-rIL-6 and FITC-A. Cells expressing the IL-6-R were obtained with fluorescence-activated cell sorter (FACS), resulting in the identification of a candidate plasmid clone, pBSF2R-236 (13). To confirm that this clone contained the cDNA encoding IL-6-R, we used murine COP cells for efficient expression. More than 10% of pBSF2R-236 transfected COP cells expressed IL-6-R as measured by B-rIL-6 binding when compared to the control cells transfected with CDM8 vector DNA. Moreover, the binding of B-rIL-6 was competitively inhibited by excess amounts of rIL-6, but not rIL-1 $\beta$  or rIL-2 (Fig. 1).

JBsf2R, a stable transfectant expressing

**Fig. 3.** RNA blot analysis of IL-6-R mRNA. Poly(A)<sup>+</sup> RNA was isolated from U266 (lanes 1, 3, and 8), U937 (lane 2), CESS (lane 4), BL29 (lane 5), Jurkat (lane 6), and YT (lane 7). RNA (10  $\mu$ g) was denatured and subjected to blotting analysis (26) using the insert cDNA of pBSF2R-236 as a probe (lanes 1 to 7). The filter was exposed for 20 min (lane 1) or 16 hours (the other lanes). The arrow indicates the specific band hybridized to pBSF2R-236 cDNA. The band corresponding to ~28S is not specific, because it disappears when the Fsp I-Esp I fragment (see legend to Fig. 4A) was used as a probe (lane 8). The number of binding sites per cell, estimated by Scatchard analysis, were 29,000, 2,800, 2,700, and 3,700 for U266, U937, CESS, and YT cells, respectively. BL29 and Jurkat cells showed no detectable IL-6 binding sites.



**Fig. 4.** (A) Restriction endonuclease cleavage map of the insert cDNAs of pBSF2R-236 and other five clones. (B) Combined nucleotide sequence and deduced amino acid sequence of the insert cDNAs of pBSF2R-236 and other five clones. Numbers at the left margin of the sequences show positions of nucleotides and amino acids, respectively. The asterisks show potential N-glycosylation sites (Asn-X-Ser/Thr); the underlined region is a presumed signal peptide; the box encircles a presumed transmembrane domain; the dots identify a possible poly(A) addition signal. Sequencing was performed by the chain termination method (2).

$\beta$ strand	S	B	C	C'	C''	D	E	F	S
C2-SF1			*	*	*				
IL-6 R	43	' 50	60	70					
PDGF R	(11)	ITATPGV--EEDNATHWLRKPAAGSH				PSWACM--	GRRGIMRSVQLHSENLYRAG		
CSF-1-R (v-fms)		ITTRIV--MGNDVV--NCGCTYPRMK--SGRLV				EPVTDYLFGVPS--RIGSIGHPTAELSDSQTENVS			
Alpha 1 B-GP (11)		ITATVA--PLSCV--DQQRGE				DFHDNYQKV--	LTENDDHIVSFQAINNSCTATN		
Fc R	(1)	ITATMEG--THNGNS--STQFHNC					KESLVPRSSSTDPRIFTHENALVGCGGHITSRVHL		
V-SFT							PSIRSQ--	VQASFTTKA-TVNGSDETRQMEQ	
Ig V kappa		ITATCGRASOSI--SNSYKAHQQKP--SGSPRLI.IYGASTRATGIP-----AISGSGSC--TEFTETSSLOSKPFLVITYQQYN							
Ig V lambda		ITATRSSTGAVTTSNYLNHQQKP--DHLFTGLIGGTNNRAPGVP-----AISGSLIG--NKAATTTGAQTHDEIIFCALWY							
Ig V heavy		ITATTVSGSTF--SNDYITVQKPP--GRCLIEWIGYVFYHGTSODTTPLRS--MTMLVDT--KNGFSERESSVTAATMYEARNI							
CD4	(1)	ITATGATASQK--KSJQCHKNSNQJ--KILGNQGSPLTM-CPSK--LNDADSRRI.WDQGNFPLITKNNLKTEPSDTIIESEVD							
Poly Ig R (11)		ITATGFTYATR-QLKKSFYKVED-----GELVI.IIDSSSEAKDPRYKG--TTLQIQSTAKEPTNTTAKHVOINNAQDITVQSGS							

Fig. 5. Alignment of IL-6-R domain to Ig superfamily protein domains. IL-6-R, CSF-1-R (27) and human Ig V kappa chain V-III region (28) sequences were aligned to several proteins of Ig superfamily (20) by inspection. (\*) Conserved patterns common to the V, C1, and C2 sets; (#) common to the V and C2 sets; (#) common to the C1 and C2 sets (20). The known locations of  $\beta$ -strands in Ig V domains are marked with bars and capital letters above the bars. The numbers above the alignment represent positions of amino acids of IL-6-R sequence. The position of the putative disulfide bridge within IL-6-R domain is indicated by S - - S.

the IL-6-R from pBSF2R.236, was established from an IL-6-R negative human T cell line, Jurkat. Scatchard plot analysis was performed by the nonlinear least squares method in the AOS/VS (Data General Corp. Operating System) version of SAS release 4.07 (SAS Institute Inc., Cary, North Carolina) and the result was consistent with two classes of IL-6-R: a high-affinity binding site [dissociation constant ( $K_d$ )  $\sim 10^{-11} M$ , number of sites per cell ( $R_1$ )  $\sim 240 \pm 190$  (SE)] and a low-affinity binding site ( $K_d \sim 10^{-9} M$ ,  $R_2 \sim 12,000 \pm 680$ ) (Fig. 2B). The myeloma cell line, U266, Scatchard plot was also consistent with there being two classes of IL-6-R with approximately the same  $K_d$  values as the IL-6-R of the JBSF2R transfectant cells (Fig. 2A). The pBSF2R.236 cDNA, therefore, can code for both high- and low-affinity binding sites, although the mechanism that determines the affinity of the IL-6-R remains to be elucidated.

The expression of IL-6-R mRNA was analyzed by RNA blots (Fig. 3). The pBSF2R.236 cDNA probe hybridized to a single species of mRNA of approximately 5000 nucleotides (nr), extracted from the YT cell line. Similar length IL-6-R mRNA was also detected in RNA extracts of the myeloma cell line U266, the histiocytic leukemia cell line U937, and the Epstein-Barr virus-transformed B cell line CESS. In fact, these cell lines had been shown to express IL-6-R (14). However, the T cell line Jurkat and the Burkitt's lymphoma cell line BL29, both of which had no detectable IL-6-R (14), had no mRNA that hybridized with the probe. The relatively high concentration of IL-6-R mRNA in U266 cells may indicate that IL-6 functions as an autocrine growth factor for myeloma cells (5).

Although pBSF2R.236 contained the insert cDNA for the coding region of the IL-

6-R mRNA, it did not contain the full mRNA sequence, as it was only 2200 nt long. To obtain the entire sequence cDNA, we probed the same cDNA library with different labeled fragments (Fig. 4A). Three additional clones contained insert cDNAs corresponding to several parts of IL-6-R mRNA. Two other cDNA clones were also isolated from a λgt10 cDNA library from U937 cells as described (2). Four of the insert cDNAs have the same 3' end followed by the poly(A) sequence (Fig. 4a). Possible poly(A) addition signals (ATAAAA) are at positions corresponding to residues 161 and 186, 200 nt upstream of the poly(A) sequence, a position that corresponds to those previously described (15).

The nucleotide sequence (Fig. 4B) was confirmed by data on the independent clones. There is a single open reading frame, which is included in the cDNA of pBSF2R.236. In this frame, the initiator ATG, which conforms to the described criteria (16), is followed by 467 codons before the termination triplet TAG. A hydropathy plot (17) of the deduced amino acid sequence of IL-6-R showed two major hydrophobic regions, one located between residues 1 and 20, and the other located in the region of residues 359 to 386. The former is presumably a typical signal peptide, with the predicted cleavage site between Ala<sup>19</sup> and Leu<sup>20</sup> (18). The latter, the putative transmembrane domain, is immediately followed by positively charged residues (Arg-Phe-Lys-Lys), which may represent the stop transfer signal anchoring the IL-6-R in the membrane during biosynthesis (19). There are six potential N-linked glycosylation sites (Asn-X-Ser/Thr) (five in the extracellular domain and one in the intracellular domain).

The comparative sequence data were obtained from the National Biomedical Re-

search Foundation database (Bolt Beranek and Newman, Inc.) and Genetic Sequence Data Bank (Los Alamos National Laboratory). Homologies were found with several members of the Ig superfamily, including the Ig light chain variable (V) region, the rabbit poly-Ig receptor, the CD4 molecule and the alpha 1 B-glycoprotein (alpha 1 B-GP). Furthermore, the IL-6-R fulfills the criteria proposed by Williams and Barclay (20) for the constant 2 (C2) set of Ig superfamily as shown in Fig. 5. The IL-6-R sequence does not contain the loop (C' and C'') between the  $\beta$ -strands C and D, which is characteristic of the V set. The distance between Cys<sup>47</sup> and Cys<sup>96</sup> is 50 residues, as described for the C2 set (20). The C2 set includes several adhesion molecules, the platelet-derived growth factor (PDGF) receptor, the colony-stimulating factor 1 (CSF-1) receptor, the Fc γ receptor, and the alpha 1 B-GP (20). The receptors for peptide growth factors, such as PDGF, CSF-1, and IL-6, could then be grouped in the C2 set.

The IL-6-R lacks tyrosine kinase domains, unlike some other growth factor receptors, although IL-6 has been found to be a potent growth factor for myeloma-plasmacytoma cells (4, 5, 9). Receptors for nerve growth factor (21) and growth hormone (22) also lack the tyrosine kinase domain. The mechanism (or mechanisms) of its signal transduction for growth and differentiation could be mediated through an unknown biochemical pathway.

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13. Monkey COS7 cells were transfected with purified plasmid DNA (2.5 µg/ml) by the DEAE dextran method (2). The cells were stained with B-rlL-6 after culture for 2 days. For the preparation of B-rlL-6 (5 µg) (12) in 10 µl of 0.1M bicarbonate buffer (pH 8.4) was incubated with 1.3 µg of biotin-O-succinimide ester (Bioscience #B-1000) in dimethyl sulfoxide (DMSO) (2 mg/ml) at room temperature for 3 hours, and free biotin was separated by Sephadex G-50 column. COS7 cells ( $5 \times 10^7$ ) transfected with DNA were incubated with ~10 ng of B-rlL-6 in 10 µl of biotin-free RPMI 1640 (DeciGen RPMI 1640, Irvine Scientific) at 4°C for 90 min. The cells were washed by passing through fetal calf serum and further incubated with FITC-A at 4°C for 20 min. Positively stained cells were sorted out with a FACS 40 (Becton Dickinson) as described (23).
- Escherichia coli* MC1061/R3 was transformed with episomal DNA collected from the sorted cells as described (12). Plasmid DNA was extracted and subjected to a second round of sorting as described above. Single colonies (250) were randomly picked after four rounds of selection, and plasmid DNA was extracted from each colony. Plasmid DNA with insert cDNAs greater than 2 kb was individually sequenced into COS7 cells and the expression of the IL-6-R was analyzed by flow cytometry. COS7 cells transfected with plasmid DNA from two colonies were found to express the IL-6-R. One of them, pBSE2R.236, was extensively analyzed.
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29. Supported in part by grants from the Ministry of Education, Science and Culture. We thank M. Hatakeyama and S. Minamoto for advice, R. Kamen for COP cells, J. Yodoi for YT cells, Y. Hirai for rIL-1 and rIL-2, H. Ohashi for help of Scatchard plot analysis, E. Barsumian, H. Kikutani, and S. Akira for review of the manuscript, and M. Hisayama and K. Kubota for secretarial assistance.
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## A Monoclonal Antibody to the $\alpha$ Subunit of $G_k$ Blocks Muscarinic Activation of Atrial K<sup>+</sup> Channels

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The activated heterotrimeric guanine nucleotide binding (G) protein  $G_k$ , at subpicomolar concentrations, mimics muscarinic stimulation of a specific atrial potassium current. Reconstitution studies have implicated the  $\alpha$  and  $\beta\gamma$  subunits as mediators, but subunit coupling by the endogenous G protein has not been analyzed. To study this process, a monoclonal antibody (4A) that binds to  $\alpha_k$  but not to  $\beta\gamma$  was applied to the solution bathing an inside-out patch of atrial membrane; the antibody blocked carbachol-activated currents irreversibly. The state of the endogenous  $G_k$  determined its susceptibility to block by the antibody. When agonist was absent or when activation by muscarinic stimulation was interrupted by withdrawal of guanosine triphosphate (GTP) in the presence or absence of guanosine diphosphate (GDP), the effects of the antibody did not persist. Thus, monoclonal antibody 4A blocked muscarinic activation of potassium channels by binding to the activated G protein in its holomeric form or by binding to the dissociated  $\alpha$  subunit.

**C**OUPLING BETWEEN THE ATRIAL muscarinic acetylcholine receptor and the potassium channel current ( $K_{ACh}$ ) that it activates (1) is thought to be independent of a cytoplasmic second messenger pathway (2). G proteins were implicated as coupling agents because whole-cell  $K_{ACh}$  currents required guanosine triphosphate (GTP), were decreased by pertussis toxin (PTX) (3), and became independent of ligand in the presence of the nonhydrolyzable GTP analog 5'-guanylylimidodiphosphate (GppNHp) (4). The muscarinic effects on the atrial membrane potential and atrial pacing were also blocked by PTX (5). Taken together with the results in (2), the

possibility of G protein mediation independent of cyclic nucleotide second messengers was noted (3). Analysis of the whole-cell currents may be complicated, however, by the presence of a PTX-insensitive nonselective cation current related to phosphoinositide hydrolysis (6). The demonstration that another nonhydrolyzable GTP analog, gua-

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**Fig. 1.** "Dot blots" of the reaction between mAb 4A and 4H (17) and transducin holoprotein  $G_i(\alpha\beta\gamma)$  (lanes 1 and 6),  $\alpha_i\beta\gamma$ S (lanes 2 and 7), the  $\beta\gamma$  subunit of transducin ( $\beta\gamma$ ) (lanes 3 and 8),  $\alpha_i$ -GTP $\gamma$ S (lanes 4 and 9), and control protein (trypsin inhibitor) (lanes 5 and 10). Dot blots were performed as described in (12), and antibody binding was detected by  $^{125}$ I-labeled protein A binding and autoradiography of dot blots. The mAb 4A has a similar affinity for  $\alpha\beta\gamma$  and  $\alpha_i$ , lower affinity for  $\beta\gamma$ , and no affinity for  $\alpha_i$ .

	αβγ	α <sub>i</sub>	βγ	α <sub>k</sub>	Ctrl	αβγ	α <sub>i</sub>	βγ	α <sub>k</sub>	Ctrl
ng										
40	●	●								
20	■	■								
10	●	●								
5	●	●								
2.5	■	■								
1.25	■	■								
0.62										
0.31										
	1	2	3	4	5	6	7	8	9	10

# **EXHIBIT D**

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## Interleukin-6 Triggers the Association of Its Receptor with a Possible Signal Transducer, gp130

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### Summary

Interleukin-6 mediates pleiotropic functions in various types of cells through its specific receptor (IL-6-R), the cDNA of which has already been cloned. We report here that an 80 kd single polypeptide chain (IL-6-R) is involved in IL-6 binding and that IL-6 triggers the association of this receptor with a non-ligand-binding membrane glycoprotein, gp130. The association takes place at 37°C within 5 min and is stable for at least 40 min in the presence of IL-6, but does not occur at 0°C. Human IL-6-R can associate with a murine gp130 homolog and is functional in murine cells. Mutant IL-6-R lacking the intracytoplasmic portion is functional, suggesting that the two polypeptide chains interact to involve their extracellular portion. In fact, a soluble IL-6-R lacking the transmembrane and intracytoplasmic domains can associate with gp130 in the presence of IL-6 and mediate its function. These findings indicate that the complex of IL-6 and IL-6-R can interact with a non-ligand-binding membrane glycoprotein, gp130, extracellularly and can provide the IL-6 signal.

### Introduction

IL-6 is a multifunctional cytokine active on B cells, T cells, plasmacytomas, hepatocytes, and several other types of cells (Hirano and Kishimoto, 1989). IL-6 has been reported to be involved in:

- induction of immunoglobulin production in activated B cells (Hirano et al., 1985, 1986)
- induction of proliferation of hybridoma/plasmacytoma/myeloma cells (Van Snick et al., 1986, 1987; Nordan and Potter, 1986; Kawano et al., 1988)
- induction of IL-2 production, cell growth, and cytotoxic T cell differentiation of T cells (Garman et al., 1987; Lotz et al., 1988; Okada et al., 1988)
- stimulation of multipotent colony formation in hematopoietic stem cells (Ikebuchi et al., 1987)
- regulation of acute phase response (Andus et al., 1987; Gauldie et al., 1987)
- growth inhibition and induction of differentiation into macrophages of myeloid leukemic cell lines (Miyaura et al., 1988)
- induction of neural differentiation (Satoh et al., 1988). In accordance with multifunctional properties of IL-6, the

specific receptor for IL-6 was found to be expressed on a variety of cells, although its number was extremely low (Taga et al., 1987). The cDNA for human IL-6-R was cloned and the cDNA was shown to have a capacity to express both high and low affinity binding properties that were similar to the naturally expressed IL-6-R on a myeloma cell line, U266 (Yamazaki et al., 1988). The nucleotide sequence of the cDNA shows that IL-6-R consists of ~449 amino acids with an intracytoplasmic portion of ~82 amino acid residues.

Although IL-6 was shown to be a potent growth factor for hybridoma/plasmacytoma/myeloma cells and a certain T cell line (Van Snick et al., 1986, 1987; Nordan and Potter, 1986; Kawano et al., 1988; Shimizu et al., 1988), the intracytoplasmic portion of IL-6-R has no tyrosine kinase domain, unlike certain other growth factor receptors (Ulrich et al., 1984, 1985; Yarden et al., 1985), or shows no homology with other known functional proteins. Thus it is suggested that transduction of the IL-6 signal could be mediated through another molecule associated with the IL-6-R. In this study, we report that a single 80 kd polypeptide chain, whose cDNA has been previously cloned, is the only surface molecule involved in the binding of IL-6. The present study shows that IL-6 triggers the association of the 80 kd IL-6-R and another non-ligand-binding membrane glycoprotein, gp130, which is most likely involved in the signal transduction. The interaction of two membrane polypeptide chains, a ligand-binding chain, and a signal-transducing chain, may be a novel mechanism for cytokine-mediated signal transduction.

### Results

#### A Single 80 kd Polypeptide Chain (80K IL-6-R) Is Responsible for IL-6 Binding

Anti-IL-6-R antibodies were prepared with a synthetic peptide made on the basis of the deduced amino acid sequence of the cytoplasmic portion of the IL-6-R (Figure 1, peptide-1). As shown in Figure 2A, anti-peptide-1 antibody precipitated a polypeptide chain with a relative molecular weight of 80,000 from an IL-6-R-positive human myeloma cell line, U266 (lane 1). No molecules were precipitated from an IL-6-R-negative human T cell line, Jurkat (lane 2), but a band with a similar relative molecular weight was detected in Jurkat transfected JIL6R with IL-6-R cDNA, indicating that this antibody was specific to IL-6-R (lane 3). Some faint bands of 50 kd and 120 kd are nonspecific, since these bands were also observed with nonimmune rabbit serum (data not shown). The relative molecular weight of the IL-6-R from U266 and JIL6R was slightly different (80,000 from U266, 84,000 from JIL6R; lanes 1 and 3). This seems to be due to a difference in sugar modification, since the relative molecular weight of the immature form of IL-6-R from both cells was found to be the same by pulse labeling and immunoprecipitation experiments (data not shown).

To detect the IL-6 binding molecule, metabolically la-

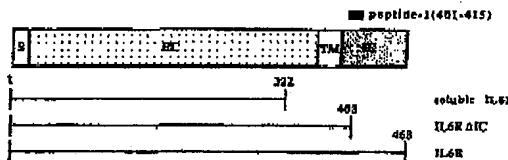
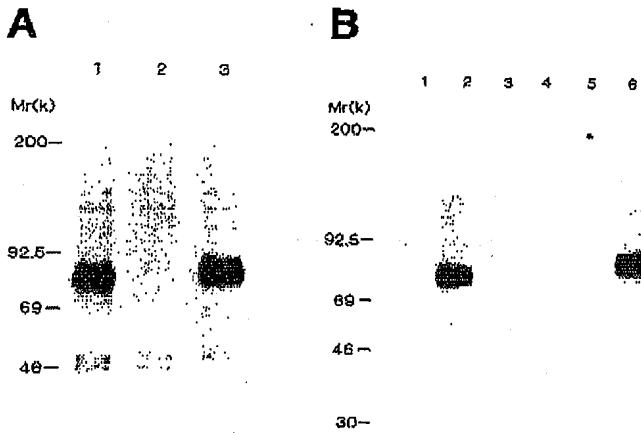
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Figure 1. Structures of IL-6-R Mutants

Structures of native and mutant IL-6-R are shown. Numbers correspond to the positions of amino acids reported by Yamasaki et al. (1989). A peptide used for immunization is also indicated as a solid bar. S, signal sequence; EC, extracellular domain; TM, transmembrane domain; IC, intracytoplasmic domain.

beled U266, Jurkat, and JIL6R cells were saturated with unlabeled IL-6 and cross-linking was performed with a cleavable cross-linker, EG5. Extracted proteins were denatured and the molecules cross-linked to IL-6 were immunoprecipitated twice to reduce nonspecific precipitates by affinity-purified polyclonal anti-IL-6 antibody. Intermolecular cross-linking bonds in the immunoprecipitates were cleaved by hydroxylamine, and the samples were analyzed by SDS-PAGE. As shown in Figure 2B, only a protein species with a relative molecular weight of about 80,000 was detected in U266 and JIL6R (lanes 2 and 6). No such band was observed in the same experiment without IL-6 (lanes 1 and 5), or in an IL-6-R-negative cell line, Jurkat, with or without IL-6 (lanes 3 and 4). The faint band of 120 kd is nonspecific, since this molecule was also precipitated in the absence of IL-6 (lanes 1, 3, and 5) even though the immunoprecipitation was performed utilizing anti-IL-6 antibody. These observations indicated that the 80K IL-6-R was the only molecule involved in the IL-6 binding.



rose beads. Immunoprecipitates were eluted by boiling the beads for 5 min in 2% SDS, 62.5 mM Tris (pH 6.8) and eluates were diluted four times with 0.5% NP40, TBS. Re-immunoprecipitation was performed with 10 µg of the same antibody and cross-linking bonds in the precipitated complexes were cleaved by incubating in 0.1 M hydroxylamine at 37°C for 12 hr and analyzed under reducing conditions on SDS-PAGE. Lanes 1 and 2, U266; lanes 3 and 4, Jurkat; lanes 5 and 6, JIL6R.

### IL-6 Stimulation Triggers the Association of 80K IL-6-R and gp130

To examine the possible presence of a signal-transducing molecule associated with the IL-6-R, the IL-6-R was precipitated under a nondissociating condition with the monoclonal anti-IL-6-R antibody, MT18, which showed less nonspecific binding property than the anti-peptide antibody. As shown in Figure 3A (lanes 1 and 3), MT18 precipitated the 80K IL-6-R from U266 cells under mild lysis conditions (1% digitonin buffer). Another polypeptide chain of 130 kd was coprecipitated when the cells were incubated with 1 µg/ml of IL-6 at 37°C for 30 min before digitonin lysis (lanes 2 and 4). The same results were obtained by SDS-PAGE under either reducing or nonreducing conditions. These observations suggested that IL-6 triggered the association of the 80K IL-6-R with a cellular 130 K molecule.

As shown in Figure 3B, varying concentrations of IL-6 were used to examine the IL-6-triggered association of IL-6-R and the 130K protein at 37°C or at 0°C. The association was observed in the cells incubated with IL-6 at concentrations of 0.04, 0.4, and 4 µg/ml at 37°C for 30 min (lanes 2, 3, and 4) but was absent in cells incubated with 4 µg/ml IL-6 at 0°C (lane 5). The kinetics of the association of IL-6-R and the 130K protein were further examined. U266 cells were surface-labeled and incubated with 1 µg/ml IL-6 at 37°C for varying periods of time, then digitonin lysis and immunoprecipitation with MT18 were performed. A 5 min incubation with IL-6 (Figure 3C, lane 2) was shown to be sufficient for the association of IL-6-R and the 130K protein, and the complex was stable for at least 40 min (lane 5) in the presence of IL-6. Furthermore, since the cells were surface-labeled, the findings indicated that the 130K protein is a cell-surface molecule.

To examine whether the 130K molecule is a glycoprotein, the U266 cells were surface-labeled and incubated

Figure 2. Immunoprecipitation of IL-6-R

(A) Immunoprecipitation of IL-6-R by anti-peptide antibody. Metabolically labeled cells ( $10^7$ ) were lysed in NP40 extraction buffer and immunoprecipitation was performed with affinity-purified anti-peptide-1 antibody as described in Experimental Procedures. Samples were analyzed under reducing conditions on SDS-PAGE with  $^{14}$ C-labeled markers (Amersham). Lane 1, U266; lane 2, Jurkat; lane 3, IL-6-R-cDNA transfected Jurkat, JIL6R.

(B) Detection of the IL-6-binding molecule by cross-linking, immunoprecipitation, and cleavage of the cross-linker. Metabolically labeled cells ( $10^7$ /lane using 0.5 mCi [ $^{35}$ S]methionine) were saturated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) unlabeled IL-6 and cross-linking was processed as described in Experimental Procedures. NP40 lysates were denatured by boiling in 0.5% SDS for 5 min, and cross-linked complexes containing IL-6 were precipitated by 10 µg of affinity-purified rabbit anti-IL-6 antibody and protein A-Sepharose.

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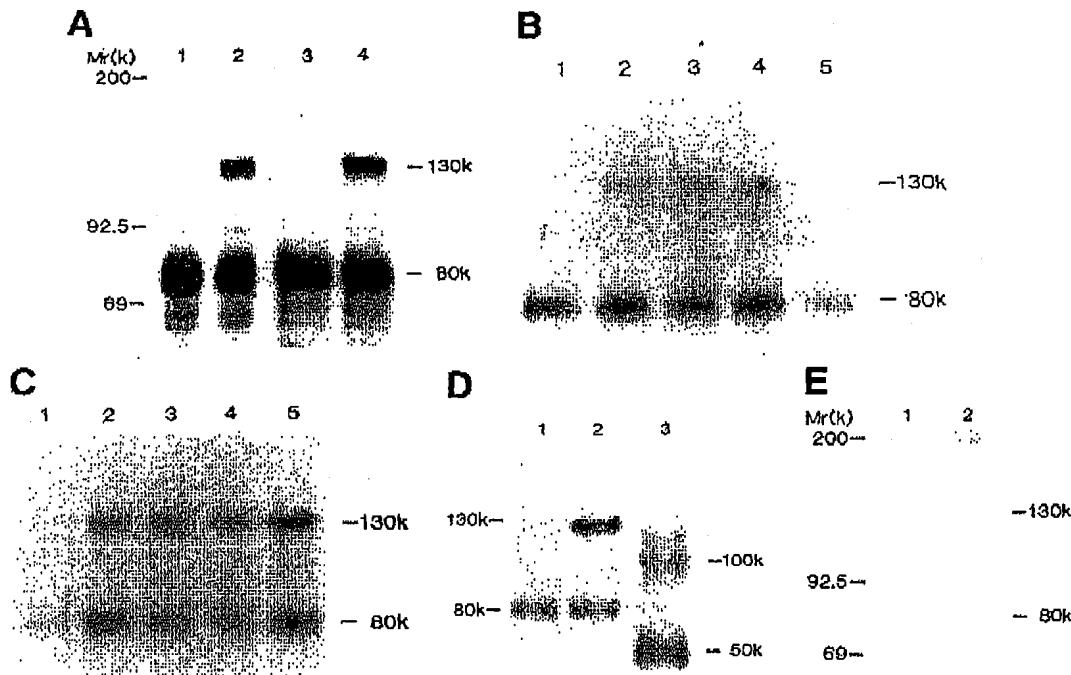


Figure 3. IL-6-Triggered Association of 80K IL-6-R and a Membrane Glycoprotein, gp130

(A) IL-6-induced association of IL-6-R and 130K protein. U266 cells ( $2 \times 10^7$ ) were metabolically labeled with 1 mCi [ $^{35}$ S]-methionine and each half was incubated with (lanes 2 and 4) or without (lanes 1 and 3) 1  $\mu$ g/ml IL-6 for 30 min at 37°C in 0.5 ml RPMI1640 medium. Digitonin lysis and immunoprecipitation with MT18 antibody were performed as described in Experimental Procedures and analyzed under nonreducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions by SDS-PAGE.

(B) Association of IL-6-R and 130K protein in the presence of varying concentrations of IL-6 at 37°C or at 0°C. U266 cells ( $2.5 \times 10^7$ ) were metabolically labeled with 1.25 mCi [ $^{35}$ S]-methionine and divided into five aliquots. Each aliquot was incubated without (lane 1) or with 0.04 (lane 2), 0.4 (lane 3), and 4  $\mu$ g/ml (lanes 4 and 5) IL-6 in 0.5 ml RPMI1640 medium at 37°C (lanes 1–4) or 0°C (lane 5) for 30 min. Digitonin lysis and immunoprecipitation were performed as above and analyzed under reducing conditions by SDS-PAGE.

(C) Kinetics of the association of IL-6-R and 130K protein. U266 cells ( $2.5 \times 10^7$ ) were surface-labeled with 2.5 mCi Na [ $^{125}$ I] as described in Experimental Procedures and divided into five aliquots. Each aliquot was mixed with (lanes 2–5) or without (lane 1) 1  $\mu$ g/ml IL-6 in 0.5 ml RPMI1640 medium and incubated for 5, 10, 20 (lanes 2, 3, and 4, respectively), and 40 min (lanes 1 and 5) at 37°C. Digitonin lysis and immunoprecipitation were performed as above and analyzed under reducing conditions by SDS-PAGE.

(D) Endoglycosidase digestion of IL-6-R and 130K protein. U266 cells ( $2 \times 10^7$ ) were surface-labeled using 2 mCi Na [ $^{125}$ I] and each half was incubated with (lane 2) or without (lane 1) 1  $\mu$ g/ml IL-6 for 30 min at 37°C and solubilized with 0.4 ml 1% digitonin lysis buffer. Immunoprecipitation was performed with 10  $\mu$ g MT18 and half aliquots were analyzed under reducing conditions by SDS-PAGE. A remaining aliquot analyzed in lane 2 was further processed by serial treatment with neuraminidase and N- and O-glycanases (lane 3) as described in Experimental Procedures.

(E) Association of IL-6-R and gp130 on HepG2 cells. HepG2 cells ( $10^7$ ) were surface-labeled with 1 mCi Na [ $^{125}$ I] and each half was incubated with (lane 2) or without (lane 1) 1  $\mu$ g/ml IL-6 for 30 min at 37°C. Digitonin lysis and immunoprecipitation were processed. Samples were analyzed under reducing conditions on SDS-PAGE.

with IL-6. The IL-6-R and the 130K protein were coprecipitated with MT18 (Figure 3D, lane 2) and further treated with neuraminidase, and with N- and O-glycanases to remove the sugar moieties and analyzed by SDS-PAGE (lane 3). Two major bands of 50 kd and 100 kd are shown in lane 3, indicating that the 80K IL-6-R molecule and the 130K molecule are glycoproteins having core proteins of, respectively, 50 kd and 100 kd. The 50K value agrees well with the calculated molecular weight based on the amino acid sequence deduced from the IL-6-R cDNA (Yamasaki et al., 1988). The other faint band of 120 kd might correspond to a partially deglycosylated 130K molecule. These results indicated that the 130K molecule was a membrane glycoprotein (gp130).

gp130 was detected in other cells responsive to IL-6. The human hepatocyte cell line HepG2 secretes several acute phase proteins following IL-6 stimulation (Gauldie et al., 1987). In Figure 3E, HepG2 cells were surface-labeled and incubated in the presence or absence of IL-6, then digitonin lysis and immunoprecipitation were processed. The gp130 was detected in this cell line (lane 2).

#### Human IL-6-R Can Associate with Murine gp130 and Is Functional in a Murine B Lymphoma Line

Human IL-6-R cDNA was transfected to a murine B lymphoma line, M12, which has no detectable number of IL-6-R (Table 1), and M12IL6R transfeciant was obtained. M12IL6R was found to possess 1800 specific IL-6-binding

Table 1. IL-6-R on Murine Transfectants and Parental Cells			
Cell	Human IL-6-R <sup>a</sup>	ShearCell <sup>b</sup>	Kd (pM) <sup>b</sup>
M12	—	ND	—
M12IL6R	IL-6-R	1800	210
M1	—	50	380
M1IL6R	IL-6-R	1000	330
M1IL6RA IC	IL-6-RΔ IC	2300	920

<sup>a</sup> The structure of human IL-6-R cDNA introduced in the transfectants is shown in Figure 1.

<sup>b</sup> Scatchard analysis was performed using 1 pM–0.8 nM  $^{125}$ I-IL-6. No significant high affinity binding sites were observed because of a relatively low number of total binding sites.

ND: not detected.

sites per cell (Table 1). This transfectant was tested for the expression of murine gp130 homolog, which could be associated with human IL-6-R by IL-6 stimulation. As shown in Figure 4A (lane 2), murine gp130 could be co-immunoprecipitated with the human IL-6-R by MT18 antibody when the cells were incubated with IL-6. Although M12 cells showed no responsiveness to IL-6 (Figure 4B, upper panels), M12IL6R cells with human IL-6-R cDNA responded to IL-6 with a decrease in growth at low cell density (Figure 4B, lower panels).

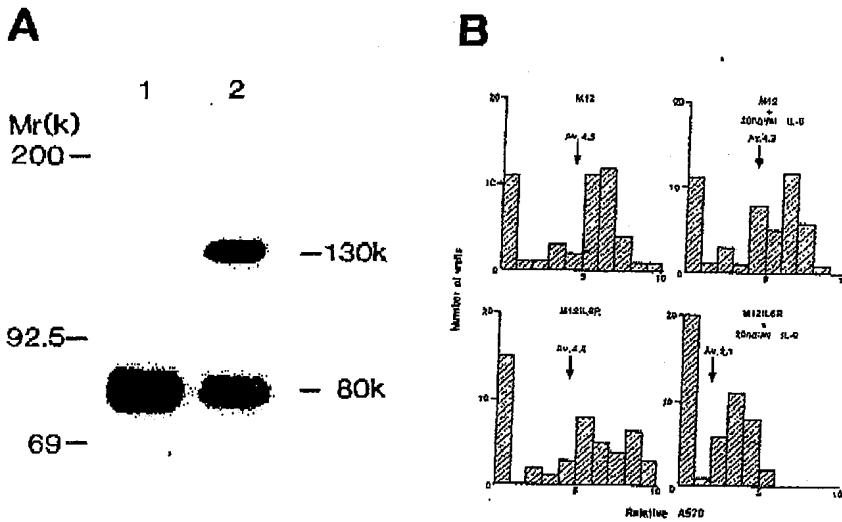
#### Intracytoplasmic Portion of IL-6-R Is Not Important for IL-6 Signal Transduction

It was examined whether the intracytoplasmic portion of IL-6-R was required for the signal transduction using the murine myeloid leukemic cell line M1, in which IL-6 in-

hibited its cell growth and induced differentiation into macrophages (Miyauchi et al., 1988). M1 cells were transfected with human IL-6-R cDNA or a deletion mutant of IL-6-R cDNA lacking the C-terminal 85 amino acid residues in the intracytoplasmic portion (Figure 1, IL6RA IC). The M1IL6R and M1IL6RA IC transfectants expressed a relatively higher number (20- to 40-fold) of IL-6 binding sites than the original M1 cells (Table 1). Cytofluorometric analysis was performed using phycoerythrin-conjugated MT18. No staining was observed with M1, but both M1IL6R and M1IL6RA IC were stained and the staining intensity was comparable for the two transfectants, indicating the expression of human IL-6-R on M1 transfectants (data not shown). These three cell lines were used to examine the responsiveness to IL-6. Cells were cultured in the presence of varying concentrations of IL-6 for 70 hr and pulse-labeled with [ $^3$ H]TdR for the last 10 hr. As shown in Figure 5, both M1IL6R and M1IL6RA IC acquired a sensitivity about 70-fold higher than M1 in their growth inhibition, indicating that human IL-6-R on murine M1 cells could transduce the IL-6 signal even with a truncated intracytoplasmic region. The M1 transfectant with CDM8 vector without an insert cDNA showed no increase in IL-6 binding sites, and sensitivity of this mock transfectant to IL-6 in growth inhibition was the same as the parent M1 (data not shown).

#### Soluble IL-6-R Can Associate with gp130 In the Presence of IL-6 and Transduce the IL-6 Signal

To examine the possibility that the extracellular domain of IL-6-R is responsible for the interaction with gp130 and the signal transduction, mutant IL-6-R cDNA coding the first 322 amino acid residues, thus lacking transmembrane



**Figure 4. Association of Human IL-6-R and Murine gp130 and IL-6-Mediated Growth Inhibition of Murine Cells through Human IL-6-R.**  
 (A) Association of human IL-6-R and murine gp130. M12IL6R (Table 1) cells (10<sup>7</sup>) were surface-iodinated and processed as in Figure 3E. Lane 1, incubated without IL-6; lane 2, with 1  $\mu$ g/ml IL-6 for 30 min at 37°C before digitonin lysis.  
 (B) Growth inhibition of M12IL6R by IL-6. M12 and M12IL6R cells were seeded in microculture plates (30 cells/ml, 0.1 ml/well) and cultured for 10 days with or without 20 ng/ml IL-6 (48 wells each). Colorimetric assay was performed as described in Experimental Procedures. Relative values of A570 of each well were expressed in a histogram. Average values (Av.) of relative A570 were also indicated.

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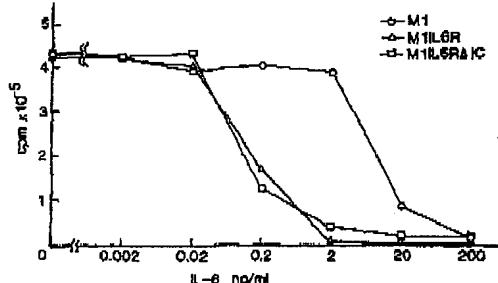


Figure 5. Intracytoplasmic Domain of IL-6-R is Not Required for Signal Transduction.

M1, M1IL6R, and M1IL6RA IC cells were cultured in microplates in the presence of varying concentrations of IL-6 and pulse-labeled with [<sup>3</sup>H]TdR as described in Experimental Procedures. Incorporated radioactivity was measured. Data represent the average of duplicate values.

and cytoplasmic domains, was prepared (Figure 1). COS7 cells were transfected with the mutant cDNA and the culture supernatant containing transiently expressed soluble IL-6-R was collected. To confirm that the soluble IL-6-R could bind IL-6, the soluble IL-6-R was applied on MT18-coated microtiter wells and the wells were incubated with [<sup>125</sup>I]IL-6. Mock control could not bind [<sup>125</sup>I]IL-6 while soluble IL-6-R could, and this binding was inhibited by excess amount of unlabeled IL-6, indicating that the binding was IL-6-specific (data not shown). To examine whether the non-membrane-anchored soluble IL-6-R could associate with gp130 in the presence of IL-6, the soluble IL-6-R was

mixed with surface-iodinated M12 (a murine B lymphoma line) cells and incubated with or without IL-6; then digitonin lysis and immunoprecipitation with MT18 were processed. As shown in Figure 6A, gp130 was detected by SDS-PAGE from the cells incubated with soluble IL-6-R plus IL-6 (fourth lane from the left), indicating that the soluble IL-6-R could associate with gp130 in the presence of IL-6. The coimmunoprecipitation of gp130 with the soluble IL-6-R was not observed in the absence of IL-6 (third lane) or with the mock control plus IL-6 (second lane). A similar result was also observed with M1 cells, in which soluble IL-6-R and gp130 could be co-immunoprecipitated when the cells were incubated with IL-6 (data not shown).

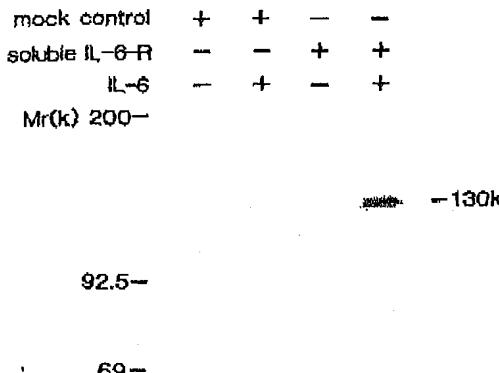
To study whether IL-6 and soluble IL-6-R could transduce the signal, M1 cells, which are more sensitive to IL-6 than M12 cells, were used in the growth inhibition assay. The cells were incubated with soluble IL-6-R or mock control in the presence or absence of varying concentrations of IL-6 for 70 hr and pulse-labeled with [<sup>3</sup>H]TdR for the last 10 hr. As shown in Figure 6B, soluble IL-6-R had no influence on M1 in the absence of IL-6 (second column from the left), but it augmented the sensitivity of M1 cells to IL-6 in their growth inhibition as compared with mock controls, indicating that the complex of soluble IL-6-R and IL-6 could transduce IL-6 signal.

#### Discussion

#### Association of a Ligand-Binding 80K IL-6-R and a Non-Ligand-Binding gp130 Molecule

We have demonstrated here that the 80K IL-6-R is the only molecule involved in ligand binding and that IL-6-R associates with a possible signal transducer, gp130, in the

#### A



#### B

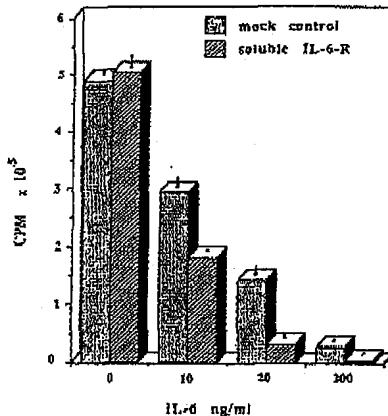


Figure 6. Soluble IL-6-R Associates with gp130 In the Presence of IL-6.

(A) Association of soluble IL-6-R and gp130 in the presence of IL-6. M12 cells ( $2 \times 10^7$ ) were surface-iodinated with 1 mCi Na[<sup>125</sup>I]. Each  $5 \times 10^6$  cell aliquot was incubated with or without 1  $\mu$ g/ml IL-6 in 1 ml of COS7 culture supernatant containing soluble IL-6-R or mock control as indicated above the figure for 30 min at 37°C. Digitonin lysis and immunoprecipitation with MT18 was processed as described in Experimental Procedures.

(B) Growth inhibition of M1 cells by soluble IL-6-R plus IL-6. M1 cells were cultured in microplates ( $1 \times 10^6$  cells/ml, 0.2 ml/well) with indicated concentrations of IL-6 in the presence of 25% COS7 culture supernatant containing soluble IL-6-R or mock control. [<sup>3</sup>H]TdR was added at 60 hr through 70 hr of the culture and the incorporated radioactivity was measured. Data represent the average (columns) of triplicate measurements with SD (vertical bars).

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presence of IL-6. Immunoprecipitation of metabolically labeled cellular proteins by anti-peptide or monoclonal anti-IL-6-R antibodies under dissociating/nondissociating conditions and SDS-PAGE under reducing/nonreducing conditions detected a single 80K molecule (Figure 2A; Figure 3A, lanes 1 and 3). The existence of another component of the IL-6-R besides the 80K molecule was unlikely, since no significant molecules other than the 80K IL-6-R were detected in the experiment shown in Figure 2B, in which the molecules cross-linked to IL-6 were collected by anti-IL-6 antibody and analyzed by SDS-PAGE after the cleavage of intermolecular cross-linking bonds.

Association of IL-6-R and the second polypeptide chain, gp130, was observed by incubating the cells with IL-6 at 37°C within 5 to 40 min (Figure 3C), but not at 0°C in 30 min (Figure 3B, lane 5), although it was previously shown that IL-6 can bind to the receptor at 0°C (Taga et al., 1987). Thus the results indicate that the association can occur at 37°C but not at 0°C even when IL-6 is bound to IL-6-R. The use of 40 ng/ml IL-6 was sufficient for the association and no increase in the association was observed with increased amounts of IL-6 up to 4 µg/ml (Figure 3B). The specific activity of the recombinant IL-6 was previously shown to be 200 pg/U (Hirano et al., 1988) and the optimum concentration of IL-6 required for stimulation of HepG2 cells for fibrinogen production was 80 U/ml (i.e., 12 ng/ml) (Gauldie et al., 1987). Thus the results indicate that association of IL-6-R and gp130 could occur in a physiological concentration of IL-6 and might not be an artifact caused by an extreme dose of IL-6. A difference in the ratio of IL-6-R and gp130 among U266 and HepG2 was observed (Figure 3D, lane 2; Figure 3E, lane 2). It could suggest that there might be a difference in the amount of 80K IL-6-R and gp130 expressed on the various cells. gp130 was also observed on murine transfectant M12/IL6R cells (Figure 4). The gp130 molecule could be induced on this transfector by the presence of IL-6-R mRNA, but this possibility can be excluded since gp130 was coprecipitated also from surface-iodinated, IL-6-stimulated parental M12 cells with soluble IL-6-R and MT18 antibody (Figure 6A, fourth lane).

M12 cells had no detectable binding capacity to [<sup>125</sup>]IL-6 (Table 1) while they did express gp130 (Figure 4A, lane 2; Figure 6A, fourth lane), confirming that the association molecule gp130 did not have a ligand-binding property by itself. Moreover, M12 cells could not bind [<sup>125</sup>]IL-6 even at 37°C (data not shown). These observations suggest that the IL-6-R system is composed of two functional chains: the ligand-binding 80K IL-6-R and the non-ligand-binding but signal-transducing gp130. This may be in contrast to the IL-2-R system. In fact, IL-2-R was shown to consist of two membrane polypeptide chains, p55 (α chain, Tac) and p75 (β chain), both of which have IL-2 binding properties (Sharon et al., 1986; Taudo et al., 1986; Teshigawara et al., 1987), but only the β chain was shown to mediate IL-2 signal (Robb and Green, 1987; Hatakeyama et al., 1987).

#### gp130 Is a Possible Signal Transducer

The findings support the possibility that gp130 is involved in IL-6 signal transduction: First, all the IL-6-responsive

cells tested, including HepG2, CESS, and KT3, expressed gp130 (Figure 3E and data not shown). These cells respond to IL-6 to produce acute phase proteins, to secrete IgG, and to promote cell growth (Gauldie et al., 1987; Hirano et al., 1985; Shimizu et al., 1988). Second, IL-6-R-negative murine M12 cells acquired responsiveness to IL-6 after the expression of human IL-6-R cDNA (Figure 4B), and the association of human IL-6-R and murine gp130 was observed (Figure 4A). This indicated a possible conserved functional importance of the association of the two molecules in murine and human cells. Third, a mutant IL-6-R that lacked most of the intracytoplasmic portion could still transduce the signal when expressed on murine M1 cells (Figure 5). Fourth, the soluble IL-6-R could associate with gp130 in the presence of IL-6 (Figure 6A) and provided the IL-6 signal (Figure 6B). The effect of soluble IL-6-R on the growth inhibition of M1 cells was more apparent at higher dose, of IL-6 (Figure 6B), suggesting that the interaction of IL-6 and soluble IL-6-R may be weaker than the native IL-6-R, or that the amount of soluble IL-6-R contained in COS7 culture supernatant may not be enough to generate a sufficient signal. The dissociation constant ( $K_d$ ) of the interaction between IL-6 and soluble IL-6-R was measured to be 1 nM (data not shown), which is lower than that of native IL-6-R on M1 cells (Table 1). Augmented sensitivity of M1 cells to IL-6 in their growth inhibition by COS7 cell supernatant containing soluble IL-6-R was diminished by absorbing the supernatant with MT18 monoclonal antibody-coated protein A-Sepharose (data not shown). This indicated that the soluble IL-6-R present in the COS7 cell supernatant was actually active on M1 cells. The possibility may not be excluded completely that soluble IL-6-R and IL-6 may form a multimerized complex that can interact efficiently with murine IL-6-R on M1 cells, resulting in augmentation of the sensitivity to IL-6.

#### Site and Mechanism of the Association of IL-6-R and gp130

gp130 was shown to be a membrane molecule that is exposed outside the cells (Figures 3C and 3D), unlike *ras* oncogenes or G proteins, which can act as signal transducers submembranously (Neer and Clapham, 1988). The results showed that the association of IL-6-R and gp130 occurred extracellularly: non-membrane-anchored soluble IL-6-R could associate with gp130 in the presence of IL-6 (Figure 6A). Recent molecular cloning of the cDNA for murine IL-6-R showed no significant homology between IL-6-Rs of human and mouse in their intracytoplasmic regions, whereas highly homologous regions were detected extracellularly (Totsuka et al., preparation), indicating the noncritical importance of their intracytoplasmic regions in IL-6-mediated events. Although no direct physical association between IL-6-R and gp130 could be shown, gp130 can be coprecipitated with IL-6-R only following IL-6 stimulus. It remains to be seen whether a qualitative change is essential for IL-6-R and gp130 to coprecipitate. It could be possible that an allosteric change in 80K IL-6-R takes place following IL-6 binding and that this in turn can affect gp130. Alternatively, IL-6

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bound to IL-6-R might change its conformation to be recognized by gp130.

Very little is known about IL-6 signal transduction: no  $\text{Ca}^{++}$  change, PI turnover, or change in cytoplasmic pH were observed in IL-6 stimulated CESS cells (Kishi et al., unpublished data). The IL-6-R was shown not to have any unique sequence for signal transduction. The mechanism by which one ligand (IL-6) can mediate multiple functions in various tissues is of paramount interest. As suggested in this study, the presence of gp130 following IL-6-interaction with its receptor might be essential for the biological activities to take place. The finding of gp130 may provide a clue to cytokine-mediated signal transduction. There could be gp130 variants in various tissues, or a third molecule varying in each type of cell might interact with gp130. This possibility has yet to be demonstrated. Recently, IL-1-R and IFN  $\gamma$ -R were molecularly cloned (Sims et al., 1988; Aguet et al., 1988), but their intracytoplasmic portions also had no homology to any known functional structures or could not explain the action of these cytokines. The present study suggests the possible existence of a molecule like gp130 for other cytokine-receptor systems, including IL-1-R and IFN  $\gamma$ -R. It can be proposed that a molecule like gp130 could act as a signal amplifier as well as a signal transducer for a ligand whose receptors are not abundant on the cell surface.

#### Experimental Procedures

##### Transfectants

J1B1R is the same cell line as JBSF2R as shown by Yamasaki et al. (1988). M11LBR was established using human IL-6-R cDNA inserted in CD46 (Seed, 1987) by cotransfection with pSV2Neo (Southern and Berg, 1982) by electroporation. M12LBR was obtained by transfecting M12 cells with human IL-6-R cDNA inserted at the BamHI site of pZip-NeoSV (XO) (Cepko et al., 1984) by electroporation and screened with G418 (Sigma). Mutant IL-6-R cDNA coding amino acid residues 1-403, thus lacking 65 out of 82 intracytoplasmic amino acid residues (Figure 1), was prepared using a Universal Transcription Terminator (Pharmacia). This mutant cDNA was inserted in pZipNeoSV(XO) and transfected into M1 cells (kindly provided by Dr. T. Suda) by electroporation; then M11LBR A 1C was obtained after G418 screening.

##### Antibodies

A synthetic peptide was prepared on the basis of the deduced amino acid sequence of the cytoplasmic portion of the IL-6-R (Figure 1, peptide-1). Anti-peptide antisera were obtained by immunizing rabbits with the peptide coupled to ovalbumin as described by Hirano et al. (1987). Antibody was affinity-purified by a peptide column. Rabbit anti-IL-6 antibody was prepared as described by Hirano et al. (1988). MT18 is a mouse anti-human IL-6-R monoclonal antibody (Hirata et al., unpublished data). MT18 was purified from ascites by ammonium sulfate precipitation and protein A-Sepharose (Pharmacia) column chromatography.

##### Internal Labeling

Cells ( $10^7$ ) were washed in methionine-free RPMI 1640 medium and precultured in 2 ml of methionine-free RPMI1640 medium, 10% dialyzed FCS for 15 min at 37°C. Precultured cells were incubated in 0.5 ml of the same medium containing 0.5 mCi  $^{35}\text{S}$ -methionine (NEN) for 4 hr followed by a label chase with normal RPMI1640 medium for 0.5 hr.

##### Cell-Surface Labeling

Cells ( $10^7$ ) were washed in PBS and resuspended in 0.1 ml of 50 mM Tris (pH 7.4), 0.15 M NaCl,  $\text{Na}^{[35\text{S}]}$  (NEN) was incubated in 0.1 ml of the same buffer with two iodobeads (Pierce) for 5 min at room temperature, then mixed with the cell suspension according to the manufac-

turer's procedure. The reaction mixture was incubated for 30 min at room temperature with occasional agitation.

##### Cell Lysis and Immunoprecipitation

For NP40 lysis, cells ( $10^7$ ) were lysed in 0.2 ml of 0.5% NP40 in TBS (10 mM Tris (pH 7.4), 0.15 M NaCl) containing 1 mM pAPMSF (a water-soluble derivative of PMSF, Wako chemicals) by vortexing at 4°C for 20 min. Digitonin lysis was processed according to a modification of a previous report (Oettgen et al., 1986). Cells ( $10^7$ ) were suspended in 0.2 ml of digitonin extraction buffer (1% digitonin, 10 mM triethanolamine (pH 7.8), 0.15 M NaCl, 10 mM lactacetaamide, 1 mM pAPMSF) and rotated for 15 min at 4°C. Highly water-soluble digitonin (Wako chemicals) was used. Both extracts were then centrifuged at 10,000  $\times g$  for 30 min and the clear lysates were incubated with 30  $\mu$ l packed volume of protein A-Sepharose beads precoated with normal rabbit or mouse serum (100  $\mu$ l serum, 1 hr coating) for 2 hr at 4°C by rotation. NP40 lysate was denatured by boiling for 5 min with 0.5% SDS before precast. Precleared lysates were mixed with 10  $\mu$ g purified rabbit or mouse antibody and incubated for 15 hr at 4°C followed by 1 hr incubation with 15  $\mu$ l packed volume of protein A-Sepharose. Immunoprecipitates were washed six times either in 0.1% NP40, TBS (NP40 lysate) or in digitonin lysis buffer (digitonin lysate) and subjected to SDS-PAGE. Gradient polyacrylamide gels (4% to 20%) were used.

##### Cross-Linking

Metabolically labeled cells were incubated in 1 ml of 10 mg/ml BSA, RPMI1640 containing 360 nM IL-6 for 1 hr at room temperature. After three washes in ice-cold PBS, the cells were suspended in 1 ml PBS (pH 8.3) containing 0.1 mM ethyleneglycol-bis-N-hydroxy succinimide (EGS, Sigma) and rotated for 30 min at 4°C. Cross-linking by EGS was cleaved by incubating in 0.1 M hydroxylamine (pH 8.5) for 12 hr at 37°C (Abdella et al., 1979).

##### Endoglycosidase Digestion

Immunoprecipitated materials bound on protein A-Sepharose were resuspended in 0.05 ml of 0.1 M acetate buffer (pH 6.5), 10 mM CaCl<sub>2</sub> containing 0.015 U neuraminidase (Genzyme) and incubated for 1 hr, then 5  $\mu$ l of 0.1 M D-galactono- $\gamma$ -lactone and 1  $\mu$ l of 1 U/ml O-glycanase (Genzyme) were added and incubated for 16 hr. Subsequently, 0.7  $\mu$ l of 10% SDS, 32  $\mu$ l of 0.25 M sodium phosphate buffer (pH 6.6), 0.4  $\mu$ l of 1 M phenanthroline hydrate, 5  $\mu$ l of 10% NP40, and 1  $\mu$ l of 250 U/ml N-glycanase (Genzyme) were added according to a modified manufacturer's protocol and incubated for 4 hr. All enzymatic reactions were processed at 37°C.

##### Cell Proliferation Assay

To produce M12 and its transfectant: cells were cultured for 10 days in microculture plates (30 cells/ml, 0.1 ml/well). Colorimetric assay for cell growth was processed as described (Moermann, 1983); briefly 10  $\mu$ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was added to the cell culture and incubated for 4 hr at 37°C, then mixed thoroughly with 100  $\mu$ l of 0.04 N HCl in isopropanol and A570 was measured. To produce M1 and its transfectants: cells were maintained as described (Miyaura et al., 1988). Cells were cultured for 60 hr in microculture plates (1  $\times$  10<sup>6</sup> cells/ml, 0.2 ml/well) and pulse-labeled with [<sup>3</sup>H]TdR (1  $\mu$ Ci/well) for 10 hr. Cells were harvested on glass filters and cell-associated radioactivity was measured.

##### Soluble IL-6-R

Mutant IL-6-R cDNA coding amino acids 1-322 and lacking the transmembrane and cytoplasmic domains was prepared using an *in vitro* Mutagenesis System (Amersham) with a synthetic nucleotide oligomer: 5' GTCCCTCAGTCAGAACGAGGT. Thus amino acid 323 was changed to Val and the termination codon TAG was introduced at position 324. The mutant cDNA was inserted in pSVL vector (Pharmacia). Culture supernatant of COS7 cells transfected with the plasmid by calcium phosphate method (Wigler et al., 1978) was collected on day 2. Mock control was prepared using pSVL vector without the insert.

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